PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C07K 16/30, C12N 15/11, 15/85, 1/20, G01N 33/50, A61K 39/395, 45/00

A1

(11) International Publication Number: WO 00/34337

(43) International Publication Date: 15 June 2000 (15.06.00)

(21) International Application Number: PCT/US99/29415

(22) International Filing Date: 10 December 1999 (10.12.99)

(30) Priority Data:
09/209,990 10 December 1998 (10.12.98) US

(71) Applicants *(for all designated States except US)*: TSUKUBA RESEARCH LABORATORY, TOAGOSEI CO., LTD. [JP/JP]; 2, Okubo, Tsukuba-shi, Ibaraki 300-2611 (JP). PROTEIN DESIGN LABS, INC. [US/US]; 34801 Campus Drive, Fremont, CA 94555 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: CO, Man, Sung [US/US]; 10952 Wilkinson Avenue, Cupertino, CA 95014 (US). VASQUEZ, Max [US/US]; 3813 Louis Road, Palo Alto, CA 94303 (US). ASANO, Makoto [JP/JP]; Tsukuba Research Laboratory, Toagosei Co, Ltd., 2 Okubo, Tsukuba-shi, Ibaraki 300-2611 (JP). YUKITA, Ayako [JP/JP]; Tsukuba Research Laboratory, Toagosei Co., Ltd., 2 Okubo, Tsukuba-shi, Ibaraki 300-2611 (JP). MATSUMOTO, Tomoe [JP/JP]; Tsukuba Research Laboratory, Toagosei Co., Ltd., 2, Okubo Tsukuba-shi, Ibaraki 300-2611 (JP). OTA, Yoshifumi [JP/JP]; Tsukuba Research Laboratory, Toagosei Co., Ltd., 2, Okubo Tsukuba-shi, Ibaraki 300-2611 (JP). OKAMOTO, Masaji [JP/JP]; Tsukuba Research Laboratory, Toagosei Co., Ltd., 2-Okubo Tsukuba-shi, Ibaraki 300-2611 (JP). LEVITT, Daniel [US/US]; 50 Parker Avenue, San Francisco, CA 94118 (US).

(74) Agent: BAKER, Joseph, R.; Fish & Richardson P.C., Suite 1400, 4225 Executive Square, La Jolla, CA 92037 (US).

(81) Designated States: CA, CZ, FI, HU, IL, JP, KR, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HUMANIZED MONOCLONAL ANTIBODIES AGAINST VASCULAR ENDOTHELIAL CELL GROWTH FACTOR

(57) Abstract

A humanized antibody which specifically binds to Vascular Endothelial cell Growth Factor (VEGF) is disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

HUMANIZED MONOCLONAL ANTIBODIES AGAINST VASCULAR ENDOTHELIAL CELL GROWTH FACTOR

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Application Serial No. 09/209,990 filed December 10, 1998, the disclosure of which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates generally to the combination of recombinant DNA and monoclonal antibody technologies for developing novel biologics and, more particularly, for example, to the production of non-immunogenic (in humans) immunoglobulins specific for vascular endothelial cell growth factor (VEGF) and their uses *in vitro* and *in vivo*. The present invention also relates more specifically to humanized monoclonal antibodies against VEGF, polynucleotide sequences encoding the antibodies, a method of producing the antibodies, pharmaceutical compositions comprising the antibody as an active ingredient, therapeutic agents for treating diseases accompanied with angiogenesis comprising the antibody as an active ingredient, and methods of treating such diseases.

BACKGROUND OF THE INVENTION

Many diseases are accompanied by pathological angiogenesis closely related to the symptoms and etiology of the disease. Among these, the

development and progression of solid tumors are one of the most representative diseases accompanied with angiogenesis. In this disease, it is necessary for newly generated blood vessels to elongate from existing blood vessels to tumor tissues for the proliferation of tumor tissues beyond the clinically detectable size, *i.e.*, 1-2 mm in diameter (J. Folkman, J. Natl. Cancer Inst., 82:4 (1990)). Once newly generated blood vessels have reached tumor tissues, the proliferation thereof is explosively accelerated.

Diabetic retinopathy and macular degeneration are also accompanied with pathological angiogenesis in the retina, which will frequently result in blindness. In addition, angiogenesis and capillary permeability play a key role in Kaposi sarcoma (KS), the most common tumor associated with HIV-1 infection. (R. Masood *et al.*, Proc. Nat'l. Acad. Sci., 94: 979-984 (1997)). Other diseases such as chronic articular rheumatism, psoriasis, angioma, scleroderma and neovascular glaucoma are also accompanied by pathological angiogenesis, which contributes to the major symptoms in these respective diseases (J. Folkman, N. Engl. J. Med., 320: 1211 (1989)).

Vascular endothelial cells form the innermost layer of blood vessels. When vascular endothelial cells are stimulated by a growth factor, physiologically active substance, or by mechanical damage, they proliferate and angiogenesis is effected. Growth factors which directly or indirectly stimulate the proliferation of vascular endothelial cells include, for example, bFGF (basic Fibroblast Growth Factor), aFGF (acidic Fibroblast Growth Factor), VEGF (Vascular Endothelial Cell Growth Factor), PD-ECGF (Platelet-Derived Endothelial Cell Growth Factor), TNF - α (Tumor Necrosis Factor - α), PDGF (Platelet Derived Growth Factor), EGF (Epidermal Growth Factor), TGF - α (Transforming Growth Factor - α), TGF - β (Transforming Growth Factor - β) and HGF (Hepatocyte Growth Factor) (L. Diaz-Flores *et al.*, Histol. Histopath. 9:807 (1994)).

Of these growth factors, VEGF is a glycoprotein 40,000 - 45,000 Da in molecular weight and occurs as a dimer (P.W. Leung *et al.*, Science 246:1306 (1989); P.J. Keck *et al.*, Science 246:1319 (1989)). VEGF acts through binding to its receptors (Flt-1 and KDR) to thereby promote the proliferation of cells and enhance membrane permeability (C. De Vies *et al.*, Science, 255:989 (1989); B.I. Terman *et al.*, Biochem. Biophys. Res. Commun., 187:1579 (1992)). VEGF can be distinguished from other growth factors on the basis of its specificity for vascular endothelial cells. This means that VEGF receptors are present primarily in vascular endothelial cells and are expressed at very low levels, if at all, on cells other than vascular endothelial cells.

VEGF has been suggested to play a role in cancer. Many types of tumor cells secrete VEGF (S. Kondo *et al.*, Biochem. Biophys. Res. Commun., 194(3):1234 (1993)). When cancer tissue sections are stained with anti-VEGF antibody, the cancer tissues and newly generated blood vessels surrounding them are strongly stained (H. F. Dvorak *et al.*, J. Exp. Med. 174:1275 (1991); L. F. Brown *et al.*, Cancer Res., 53:4727 (1993)). Further, the proliferation of a transplanted tumor is inhibited in mice in which a VEGF receptor is hereditarily inactivated (B. Millauer *et al.*, Nature, 367:576 (1994)). An anti-VEGF neutralizing antibody has been described that exhibited antitumor activity in tumor-carrying mice (K. J. Kimet *et al.*, Nature, 362:841 (1993); S. Kondo *et al.*, Biochem. Biophys. Res. Commun., 194:1234 (1993)).

From all of the above discussions, it is believed that VEGF secreted by tumor cells plays a major role in tumor angiogenesis. In addition, a murine monoclonal antibody, MV833, against VEGF is effective in inhibiting the proliferation of tumors in human fibrosarcoma cell HT1080 - transplanted nude mice (Japanese Unexamined Patent Publication No. 9-124697).

Unfortunately, the use of murine monoclonal antibodies, such as the MV833 antibody described above, have certain drawbacks in human treatment, particularly in repeated therapeutic regimens as explained below. Mouse

monoclonal antibodies, for example, tend to have a short half-life, and lack other important immunoglobulin functional characteristics when used in humans.

Perhaps more importantly, murine monoclonal antibodies contain substantial amino acid sequences that will be immunogenic when injected into a human patient. Numerous studies have shown that, after injection of a non-human antibody, the immune response elicited by a patient against the injected antibody can be quite strong, essentially eliminating the antibody's therapeutic utility after an initial treatment. Moreover, if mouse or other antigenic (to humans) monoclonal antibodies are used to treat various human diseases, subsequent treatments with unrelated mouse antibodies may be ineffective or even dangerous in themselves, because of cross-reactivity.

While the production of so-called "chimeric antibodies" (*e.g.*, mouse variable regions joined to human constant regions) has proven somewhat successful (see, USSN 4,816,567), a significant immunogenicity problem remains. (See, LoBuglio, A.F. *et al.*, Proc. Natl. Acad. Sci. USA, 86, 4220-4224 (1989); M.N. Saleh *et al.*, Human Antibod. Hybridomas e: 19 (1992)). In addition, in general, the production of human immunoglobulins reactive with VEGF antigen with high affinity, as with many antigens, would be extremely difficult using typical human monoclonal antibody production techniques.

Thus, there is a need for improved forms of immunoglobulins specific for VEGF antigen that are substantially non-immunogenic in humans, yet easily and economically produced in a manner suitable for therapeutic formulation and other uses. The present invention fulfills these and other needs.

SUMMARY OF THE INVENTION

The present invention provides humanized monoclonal antibodies against vascular endothelial cell growth factor (VEGF); polynucleotide sequences encoding the antibodies; methods for producing the antibodies; pharmaceutical compositions comprising the antibody or polynucleotide encoding the antibody as an active ingredient; therapeutic agents for treating diseases accompanied by angiogenesis comprising the antibody or polynucleotide encoding the antibody as an active ingredient; and a method for treating such diseases.

In one embodiment, the present invention provides a humanized antibody that specifically binds to Vascular Endothelial Cell Growth Factor (VEGF). A specific humanized antibody was derived from the murine MV833 antibody. In one embodiment, the humanized antibody comprises a CDR from a sequence as set forth in SEQ ID NO:2. The humanized antibody can comprise a heavy chain variable region sequence as set forth in SEQ ID NO:8 (starting at position 1) and a light chain variable region sequence as set forth in SEQ ID NO:6 (starting at position 1).

In yet another embodiment, the present invention provides a polynucleotide sequence encoding a humanized antibody that specifically binds to Vascular Endothelial Cell Growth Factor (VEGF). The polynucleotide can comprise a sequence encoding the light chain of the humanized antibody as set forth in SEQ ID NO:1 or SEQ ID NO:5. Similarly, the polynucleotide can comprise a sequence encoding the heavy chain of the humanized antibody is as set forth in SEQ ID NO:3 or SEQ ID NO:7. In a further embodiment, the polynucleotide sequence can comprise a sequence as set forth in SEQ ID Nos: 5 and 7.

In yet a further embodiment, the present invention provides a vector comprising the polynucleotide sequence encoding the humanized antibody of the invention. The vector can be a plasmid, viral vector or expression vector.

In another embodiment, the present invention provides a host cell containing a vector having the polynucleotide sequence encoding the humanized antibody of the invention. The host cell can be prokaryotic (*e.g.*, a bacterial cell) or eukaryotic (*e.g.*, a CHO cell).

In yet another embodiment, the host cell can be used to produce the humanized VEGF antibody of the invention, comprising culturing the cell under conditions whereby the humanized antibody is expressed; and recovering the humanized antibody.

In another embodiment, the invention provides a pharmaceutical composition comprising the humanized antibody and a pharmaceutically acceptable carrier.

The invention also provides a method of inhibiting an angiogenesis-associated disorder in subject comprising administering to the subject an effective dosage (*e.g.*, a therapeutically effective amount) of a human or humanized antibody that specifically binds to VEGF. The angiogenesis-associated disorder can be a VEGF-associated disorder such as, for example, a cancer, solid tumor, diabetic retinopathy, macular degeneration, Kaposi sarcoma, chronic articular rheumatism, psoriasis, angioma, scleroderma and/or neovascular glaucoma. Exemplary cancers applicable to the methods of the invention include breast cancer, ovarian cancer, lung cancer and colon cancer. The subject can be a mammal, but is preferably a human.

In another embodiment, the invention provides a method of increasing the median progression-free survival time of a subject having a cancer, comprising administering to the subject a standard chemotherapeutic regimen and a humanized antibody that binds to VEGF, wherein the median progression-free survival time is increased compared to a subject administered the chemotherapeutic regimen without the humanized antibody.

In yet another embodiment the invention provides a method of increasing the objective response rate of a subject having a cancer comprising

administering to the subject a chemotherapeutic regimen and a humanized antibody that binds to VEGF, wherein the objective response rate is increased compared to a subject treated with the standard chemotherapeutic regimen without the humanized antibody.

In yet a further embodiment, the invention provides diagnostic methods and compositions comprising the humanized antibody of the invention. For example, the invention provides a method of diagnosing an angiogenesis-associated disorder, comprising contacting a sample with a humanized antibody that binds to VEGF; detecting binding of the antibody; and comparing the amount of binding to a control sample, wherein an increase in binding is indicative of an angiogenesis-associated disorder.

In another embodiment, the invention provides a method for determining the prognosis of a subject having an angiogenesis-associated disorder comprising contacting the subject or a sample from the subject with a humanized antibody the binds to VEGF; determining the level of VEGF polypeptide in the subject; and correlating the level with prognosis of the subject.

The present invention provides novel compositions useful, for example, in the treatment of an angiogenesis-associated disorder, the compositions contain humanized immunoglobulins specifically capable of binding to a VEGF antigen and of substantially neutralizing VEGF activity. The immunoglobulins can have two pairs of light chain/heavy chain complexes, at least one chain comprising one or more mouse complementarity determining regions (CDRs) functionally joined to human framework region segments. For example, mouse CDRs, with or without additional naturally-associated mouse amino acid residues, can be introduced into human framework regions to produce humanized immunoglobulins capable of binding to the antigen at affinity levels stronger than about 10^7 M^{-1}. These humanized immunoglobulins

will also be capable of blocking the binding of the CDR-donating mouse monoclonal antibody to VEGF.

The immunoglobulins, including epitope binding fragments and other derivatives thereof, of the present invention may be readily produced by a variety of recombinant DNA techniques, with ultimate expression in transfected cells, preferably immortalized eukaryotic cells, such as myeloma or hybridoma cells. Polynucleotides comprising a first sequence coding for humanized immunoglobulin framework regions and a second sequence set coding for the desired immunoglobulin complementarity determining regions can be produced synthetically or by combining appropriate cDNA and genomic DNA segments.

The humanized immunoglobulins may be utilized in substantially pure form in treating angiogenesis-associated disorders such as cancer, diabetic retinopathy, macular degeneration, Kaposi sarcoma, chronic articular rheumatism, psoriasis, angioma, scleroderma and neovascular glaucoma. The humanized immunoglobulins or their complexes can be prepared in a pharmaceutically accepted dosage form, which will vary depending on the mode of administration.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows an outline of steps for cloning a cDNA coding for the mouse MV833 antibody variable region.

Fig. 2 shows the base sequence and amino acid sequence of the cDNA coding for the light chain variable region of the mouse MV833 antibody. The CDRs are underlined. The first amino acid of the mature peptide is double underlined. The base sequence and translated amino acid sequence of cDNA is shown in SEQ ID NO: 1, and the amino acid sequence is shown in SEQ ID NO: 2.

Fig. 3 shows the base sequence and amino acid sequence of the cDNA coding for the heavy chain variable region of the mouse MV833 antibody. The CDRs are underlined. The first amino acid of the mature peptide is double underlined. The base sequence and translated amino acid sequence of cDNA is shown in SEQ ID NO: 3, and the amino acid sequence is shown in SEQ ID NO: 4.

Fig. 4 shows an outline of steps for constructing cDNAs coding for the variable regions of humanized MV833 antibody.

Fig. 5 shows the base sequence and amino acid sequence of the cDNA coding for the light chain variable region of the humanized MV833 antibody. The CDRs are underlined. The first amino acid of the mature peptide is double underlined. The base sequence and translated amino acid sequence of cDNA is shown in SEQ ID NO: 5, and the amino acid sequence is shown in SEQ ID NO: 6.

Fig. 6 shows the base sequence and amino acid sequence of the cDNA coding for the heavy chain variable region of the humanized MV833 antibody. The CDRs are underlined. The first amino acid of the mature peptide is double underlined. The base sequence and translated amino acid sequence of cDNA is shown in SEQ ID NO: 7, and the amino acid sequence is shown in SEQ ID NO: 8.

Fig. 7 shows the isoelectric focusing pattern of the humanized MV833 antibody.

Fig. 8 shows the affinity of the murine and humanized MV833 antibodies for VEGF.

Fig. 9 presents graphs showing the neutralizing activity of the humanized and murine MV833 antibodies on the vascular endothelial cell growth promoting activity of VEGFs, $VEGF_{121}$(Panel A) and $VEGF_{165}$(Panel B).

Fig. 10 presents graphs showing the neutralizing activity of the humanized and murine MV833 antibodies on the VEGF activity to bind to the extracellular domains of human VEGF receptors, Flt-1 (Panel A) and KDR (Panel B).

Fig. 11 shows the results of antitumor test on the humanized MV833 antibody.

Fig. 12 A and B shows the cross-reactivity of the humanized MV833 antibody with VEGFs of various mammal-derived cells.

Fig. 13 shows the permeability enhancing effect of the humanized MV833 antibody in Miles assay.

Fig. 14 shows the synthetic oligonucleotides (SEQ ID NOS. 9 to 16) prepared for the construction of the light chain variable region of humanized MV833.

Fig. 15 shows the synthetic oligonucleotides (SEQ ID NOS. 17 to 24) prepared for the construction of the heavy chain variable region of humanized MV833.

Fig. 16 shows the comparison of the murine light chain variable framework sequences to the human I2R light chain variable framework sequences indicated the % identity.

Fig. 17 shows the comparison of the murine heavy chain variable framework sequences to the human I2R heavy chain variable framework sequences indicated the percent identity.

Fig. 18 shows the effect of the anti-VEGF antibodies on ascites fluid in an animal model compared to IgG.

Fig. 19 shows the percentage of animals surviving as a result of i.v. administration of anti-VEGF antibodies compared to control.

Fig. 20 shows the effect of the anti-VEGF antibodies on the re-accumulation of ascites fluid in an animal model compared to IgG.

Fig. 21 shows the percentage of animals surviving as a result of i.v. administration of anti-VEGF antibodies 23 days after tumor cell inoculation compared to control.

Fig. 22 compares the effect of MV833 anti-VEGF antibody+taxol, MV833 anti-VEGF antibody alone, taxol alone and a control on tumor volume in an animal model.

Fig. 23 compares the effect of HuMV833 anti-VEGF antibody+taxol, HuMV833 anti-VEGF antibody alone, taxol alone and a control on tumor volume in an animal model.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, humanized immunoglobulins specifically reactive with VEGF are provided. These immunoglobulins, which have binding affinities to human VEGF of at least about 10^7 M^{-1} to 10^{10} M^{-1}, and typically 10^8 M^{-1} or 10^9 M^{-1} to 10^{10} M^{-1} or stronger, are capable, *e.g.*, of inhibiting the binding of VEGF to its receptors and/or neutralizing the biological activity of VEGF. The humanized immunoglobulins of the invention have a human immunoglobulin framework and one or more complementarity determining regions (CDR's) from a non-human immunoglobulin, typically a mouse immunoglobulin, specifically reactive with human VEGF antigen. In a preferred embodiment, one or more of the CDR's will come from the murine monoclonal antibody MV833. However, the CDRs from other antibodies that compete with MV833, block the binding of MV833 to VEGF, and/or bind to the same epitope on VEGF as MV833 may be used. For example, another monoclonal antibody is A.4.6.1. (see, Presta *et al.*, Cancer Research 57:4593 (1997)). Thus, the immunoglobulins of the present invention, which can be produced economically in large quantities, find use, for example, in the treatment of diseases accompanied with angiogenesis including

solid tumor, diabetic retinopathy, macular degeneration, Kaposi sarcoma, chronic articular rheumatism, psoriasis, angioma, scleroderma and neovascular glaucoma in human patients by a variety of techniques.

The term "epitope", as used herein, refers to an antigenic determinant on an antigen, such as a VEGF polypeptide, to which the paratope of an antibody, such as an MV833 antibody, binds. Antigenic determinants usually consist of chemically active surface groupings of molecules, such as amino acids or sugar side chains, and can have specific three-dimensional structural characteristics, as well as specific charge characteristics.

It is envisioned that the methods of the present invention can be used to treat pathologies and disorders associated with angiogenesis. Therefore, the present invention encompasses methods for ameliorating a disorder associated with VEGF, including treating a subject having the disorder, including at the site of the disorder, with an immunoglobulin, which interacts with VEGF. Generally, the terms "treating", "treatment", "inhibiting" and the like are used herein to mean affecting a subject, tissue, or cell to obtain a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or sign or symptom thereof, and/or may be therapeutic in terms of a partial or complete cure for a disorder or disease and/or adverse effect attributable to the disorder or disease. "Treating" as used herein covers any treatment of, or prevention of a disorder or disease in, a mammal, particularly a human, and includes:

(a) preventing the disease or disorder from occurring in a subject that may be predisposed to the disease, but has not yet been diagnosed as having it;

(b) inhibiting the disease or disorder, *i.e.*, arresting its development; or

(c) relieving or ameliorating the disease, *i.e.*, cause regression of the disease.

However, it should be recognized that the compositions and methods described herein, can be used to bring about a desired result (*e.g.*, a reduction in VEGF and/or angiogenesis) in the absence of a disease or disorder.

The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25kD) and one "heavy" chain (about 50-70kD). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function.

Light chains are classified as either kappa (κ) or lambda (λ). Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region and about 12 or more amino acids, with the heavy chain also including a "D" region or about 10 more amino acids. (*See*, generally, Fundamental Immunology, Paul, W., Ed., Chapter 7, pgs. 131-166, Raven Press, N.Y. (1984), which is incorporated herein by reference.)

The variable regions of each light/heavy chain pair form the antibody-binding site, also known as the paratope. The chains all exhibit the same general structure of relatively conserved framework regions joined by three hypervariable regions, also called Complementarity Determining Regions or CDR's (see, "Sequences of Proteins of Immunological Interest," Kabat, E., *et al.*, U.S. Department of Health and Human Services, (1987); and Chothia and Lesk, J. Mol. Biol., 196, 901-917 (1987), which are incorporated herein by reference). The CDR's from the two chains of each pair are aligned by the framework regions, enabling binding to a specific epitope.

As used herein, the term "immunoglobulin" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes.

The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. The immunoglobulins may exist in a variety of forms besides antibodies; including, for example, Fv, Fab, and $F(ab')_2$ as well as bifunctional hybrid antibodies (*e.g.*, Lanzavecchia *et al.*, Eur. J. Immunol. 17, 105 (1987)) and in single chains (*e.g.*, Huston *et al.*, Proc. Natl. Acad. Sci. U.S.A., 85, 5879-5883 (1988) and Bird *et al.*, Science 242, 423-426 (1988), which are incorporated herein by reference). (See, generally, Hood *et al.*, Immunology, Benjamin, N.Y., 2nd ed. (1984), Harlow and Lane, Antibodies. A Laboratory Manual, Cold Spring Harbor Laboratory (1988) and Hunkapiller and Hood, Nature, 323, 15-16 (1986), which are incorporated herein by reference.).

Chimeric antibodies are antibodies whose light and heavy chain genes have been constructed, typically by genetic engineering, from immunoglobulin gene segments belonging to different species. For example, the variable (V) segments of the genes from a mouse monoclonal antibody may be joined to human constant (C) segments, such as γ_1 and γ_3. A typical therapeutic chimeric antibody is thus a hybrid protein of the V or antigen-binding domain from a mouse antibody and the C or effector domain from a human antibody, although other mammalian species may be used.

As used herein, the term "framework region" refers to those portions of immunoglobulin light and heavy chain variable regions that are relatively conserved (*i.e.*, other than the CDR's) among different immunoglobulins in a single species, as defined by Kabat, *et al.*, op. cit. As used herein, a "human framework region" is a framework region that is substantially identical (about 85% or more) to the framework region of a naturally occurring human antibody.

As used herein, the term "humanized immunoglobulin" refers to an immunoglobulin comprising a human framework, at least one CDR from a

non-human antibody, and in which any constant region present is substantially identical to a human immunoglobulin constant region, *i.e.*, at least about 85-90%, typically at least 95% identical. Hence, all parts of a humanized immunoglobulin, except possibly the CDR's, are substantially identical to corresponding parts of one or more native human immunoglobulin sequences. For example, a humanized immunoglobulin would not encompass a chimeric mouse variable region/human constant region antibody. The general construction of humanized immunoglobulins (antibodies) has been described in, *e.g.*, Queen *et al.*, U.S. Patent No. 5,585,089 (which is incorporated herein by reference).

Humanized antibodies have at least three potential advantages over mouse and in some cases chimeric antibodies for use in human therapy. For example, the advantages may be attributed to the fact that 1) the effector portion is human and thus may interact better with the other parts of the human immune system (*e.g.*, destroy the target cells more efficiently by complement-dependent cellular cytotoxicity (CDCC) or antibody-dependent cellular cytotoxicity (ADCC)); 2) the human immune system should not recognize the framework or C region of the humanized antibody as foreign, and therefore the antibody response against such an injected antibody should be less than against a totally foreign mouse antibody or a partially foreign chimeric antibody; and 3) injected mouse antibodies have been reported to have a half-life in the human circulation much shorter than the half-life of normal antibodies (Shaw, D. *et al.*, J. Immunol, 138, 4534-4538 (1987)). Injected humanized antibodies will presumably have a half-life essentially identical to naturally occurring human antibodies, allowing smaller and less frequent doses to be given.

Polynucleotide Sequences

In one aspect, the present invention is directed to recombinant polynucleotides encoding the heavy and/or light chain CDR's from an

immunoglobulin capable of binding VEGF, such as monoclonal antibody MV833. The polynucleotides encoding these regions will typically be joined to polynucleotides encoding appropriate human framework regions. As to the human framework region, a framework or variable region amino acid sequence of a CDR-providing non-human immunoglobulin is compared with corresponding sequences in a human immunoglobulin sequence collection, and a sequence having high homology is selected. Exemplary polynucleotides, which on expression code for the polypeptide chains comprising the heavy and light chain CDR's of monoclonal antibody MV833 are included in Figs. 2 and 3. Due to codon degeneracy and non-critical amino-acid substitutions, other polynucleotide sequences can be readily substituted for those sequences, in order to provide conservative variations in the polypeptide sequence of the immunoglobulin, as detailed below.

The term "conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. Other illustrative examples of conservative substitutions include the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine, glutamine, or glutamate; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; valine to isoleucine to leucine.

Modifications and substitutions are not limited to replacement of amino acids. For a variety of purposes, such as increased stability, solubility, affinity or configuration concerns, one skilled in the art will recognize the ability to introduce, (by deletion, replacement, or addition) other modifications. Examples of such other modifications include incorporation of rare amino acids, dextra-amino acids, glycosylation sites, and cytosine for specific disulfide bridge formation. Accordingly, modification of the polynucleotide sequences of the invention by, for example, site-directed mutagenesis, genetic engineering can be used and the polynucleotide synthesized and expressed in bacteria, yeast, insect cells, tissue culture and so on. The resulting expression product will provide a polypeptide with the desired immunoglobulin sequence.

The design of humanized immunoglobulins may be carried out as follows. When an amino acid falls within one of the following categories, the framework amino acid of a human immunoglobulin to be used (acceptor immunoglobulin) is replaced by a framework amino acid from a CDR-providing non-human immunoglobulin (donor immunoglobulin):

1. the amino acid in the human framework region of the acceptor immunoglobulin is unusual for human immunoglobulin at that position, whereas the corresponding amino acid in the donor immunoglobulin is typical for human immunoglobulin in that position;
2. the position of the amino acid is immediately adjacent to one of the CDR's; or
3. the amino acid is capable of interacting with the CDRs in a tertiary structure immunoglobulin model (see, Queen *et al.*, op. cit., and Co *et al.*, Proc. Natl. Acad. Sci. USA 88, 2869 (1991), respectively, both of which are incorporated herein by reference).

For a detailed description of the production of humanized immunoglobulins see, Queen *et al.*, op. cit., and Co *et al.*, op. cit.

The polynucleotides of the invention will typically include an expression control polynucleotide sequence operably linked to the humanized immunoglobulin coding sequences, including naturally-associated or heterologous promoter regions. "Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. An expression control sequence operatively linked to a coding sequence is ligated such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. As used herein, the term "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus, expression control sequences can include

appropriate promoters, enhancers, transcription terminators, a start codon (*i.e.*, ATG) in front of a protein-encoding gene, splicing signals for introns, maintenance of the correct reading frame of that gene to permit proper translation of the mRNA, and stop codons. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. Expression control sequences can include a promoter.

The term "promoter" means a minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the gene. Both constitutive and inducible promoters, are included in the invention (see *e.g.*, Bitter *et al.*, Methods in Enzymology 153:516-544, 1987). For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage , plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used. When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the nucleic acid sequences of the invention.

Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells, but control sequences for prokaryotic hosts may also be used. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and, as desired, the collection and purification of the light chains, heavy chains,

light/heavy chain dimers or intact antibodies, binding fragments or other immunoglobulin forms may be performed by techniques known to those of skill in the art.

The nucleic acid sequences of the present invention capable of ultimately expressing the desired humanized immunoglobulins or antibodies can be formed from a variety of different polynucleotides (genomic or cDNA, RNA, synthetic oligonucleotides, etc.) and components (*e.g.*, V, J, D, and C regions), as well as by a variety of different techniques. Joining appropriate genomic and synthetic sequences is presently the most common method of production, but cDNA sequences may also be utilized (see, European Patent Publication No. 0239400 and Riechmann, L. *et al.*, Nature, 332, 323-327 (1988), both of which are incorporated herein by reference.)

Human constant region DNA sequences can be isolated in accordance with well known procedures from a variety of human cells, but preferably immortalized B-cells (see, Kabat op. cit. and WP 87/02671). The CDR's for producing the immunoglobulins of the present invention will be similarly derived from monoclonal antibodies capable of binding to VEGF and produced in any convenient mammalian source, including, mice, rats, rabbits, or other vertebrate capable of producing antibodies by well known methods. Suitable source cells for the polynucleotide sequences and host cells for immunoglobulin expression and secretion can be obtained from a number of sources, such as the American Type Culture Collection (Catalogue of Cell Lines and Hybridomas, Fifth edition (1985) Rockville, Maryland, U.S.A., which is incorporated herein by reference).

In addition to the humanized immunoglobulins specifically described herein, other "substantially homologous" modified immunoglobulins can be readily designed and manufactured utilizing various recombinant DNA techniques well known to those skilled in the art. For example, the framework regions can vary from the native sequences at the primary structure level by

several amino acid substitutions, terminal and intermediate additions and deletions, and the like. Moreover, a variety of different human framework regions may be used singly or in combination as a basis for the humanized immunoglobulins of the present invention. In general, modifications of the genes may be readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis (*see*, Gillman and Smith, Gene 8, 81-97 (1979) and Roberts S. *et al.*, Nature 328, 731-734 (1987), both of which are incorporated herein by reference.)

Homology or identity is often measured using sequence analysis software (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of homology to various deletions, substitutions and other modifications. The terms "homology" and "identity" in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same when compared and aligned for maximum correspondence over a comparison window or designated region as measured using any number of sequence comparison algorithms or by manual alignment and visual inspection.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequence for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol 48:443 (1970), by the search for similarity method of person & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection.

On example of a useful algorithm is BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, Nuc. Acids Res. 25:3389-3402 (1977) and Altschul *et al.*, J. Mol. Biol. 215:403-410 (1990), respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences,

the parameters M (reward score for a pair of matching residues; always >0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) or 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectations (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, *e.g.*, Karlin & Altschul, Proc. Natl. Acad. Sci. USA 90:5873 (1993)). One measure of similarity provided by BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a references sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

In addition, basic molecular biology techniques such as hybridization assays may be performed to determine whether a polynucleotide sequence is substantially homologous. Such assays use increasing stringency to determine the homology between two polynucleotide sequences based on their complementarity. For example, using moderately stringent or highly stringent

physiological conditions (See, for example, the techniques described in Maniatis *et al.*, 1989 Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., incorporated herein by reference), one can distinguish related from unrelated nucleotide sequences.

In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (*e.g.*, GC v. AT content), and nucleic acid type (*e.g.*, RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter.

An example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42 °C (moderate stringency conditions); and 0.1 x SSC at about 68 °C (high stringency conditions). Washing can be carried out using only one of these conditions, *e.g.*, high stringency conditions, or each of the conditions can be used, *e.g.*, for 10-15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically.

In another embodiment, polypeptide fragments comprising only a portion of the primary antibody structure may be produced, which fragments possess one or more immunoglobulin activities (*e.g.*, complement fixation activity). These polypeptide fragments may be produced by proteolytic cleavage of intact antibodies by methods well known in the art, or by inserting stop codons at the desired locations in the vectors using site-directed mutagenesis, such as after CH1 to produce Fab fragments or after the hinge region to produce $F(ab')_2$ fragments. Single chain antibodies may be produced

by joining VL and VH with a DNA linker (see Huston *et al.*, op cit., and Bird *et al.*, op cit.). Also, because like many genes the immunoglobulin-related genes contain separate functional regions, each having one or more distinct biological activities, the genes may be fused to functional regions from other genes to produce fusion proteins having novel properties.

As stated previously, the polynucleotides of the invention can be expressed in hosts after the sequences have been operably linked to (*i.e.*, positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, *e.g.*, tetracycline or neomycin, to permit detection of those cells transformed with the desired DNA sequences (see, *e.g.*, U.S. Patent 4,704, 362, which is incorporated herein by reference).

E. coli is one prokaryotic host particularly useful for cloning the polynucleotides of the present invention. Other microbial hosts suitable for use include bacilli, such as Bacillus subtilus, and other enterobacteriacea, such as Salmonella, Serratia, and various Pseudomonas species. In these prokaryotic hosts, one can also make expression vectors which will typically contain expression control sequences compatible with the host cell (*e.g.*, an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

Other microbes, such as yeast, may also be used for expression. Saccharomyces is a preferred microbial host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate

kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired.

In addition to microorganisms, mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention (see, Winnacker, From Genes to Clones, VCH Publishers, N.Y., N.Y. (1987), which is incorporated herein by reference). Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact immunoglobulins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, preferably myeloma cell lines, etc., or transformed B-cells of hybridomas. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, and enhancer (Queen *et al.*, Immunol. Rev. 89, 46-68 (1986), which is incorporated herein by reference), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Expression control sequences are promoters derived from immunoglobulin genes, SV40, Adenovirus, Bovine Papilloma Virus, cytomegalovirus and the like.

Vectors containing the polynucleotide sequences of interest (*e.g.*, the heavy and light chain encoding sequences and expression control sequences) can be transferred into host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts. (See, generally, Maniatis *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (1982), which is incorporated herein by reference.)

Once expressed, the whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention can be purified according to standard procedures in the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel

electrophoresis and the like (see, generally, Scopes, R., Protein Purification, Springer-Verlag, N.Y. (1982), which is incorporated herein by reference). Substantially pure immunoglobulins of at least about 90 to 95% homogeneity, and typically 98 to 99% or more homogeneity are used for pharmaceutical applications. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings, and the like. (See, generally, Immunological Methods, Vols. I and II, Lefkovits and Pernis, eds., Academic Press, New York, N.Y. (1979 and 1981)).

Therapeutics

The immunoglobulins of the present invention will typically find use in treating the diseases accompanied with angiogenesis including, for example, solid tumor, diabetic retinopathy, macular degeneration, Kaposi sarcoma, chronic articular rheumatism, psoriasis, angioma, scleroderma and neovascular glaucoma in human patients.

The humanized immunoglobulins and pharmaceutical compositions thereof of this invention are particularly useful for parenteral administration by injection or by gradual perfusion over time, *i.e.*, subcutaneously, intramuscularly, intravenously, intraperitoneally or intraocularly.

The compositions for parenteral administration will commonly comprise a solution of the immunoglobulin or a cocktail thereof dissolved in an acceptable carrier of either an aqueous or non-aqueous solution, such as for example, water, buffered water, 0.4% saline, 0.3% glycine, 5% glucose, human albumin solution, alcoholic/aqueous solutions, emulsions or suspensions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's intravenous vehicles include

fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents and inert gases and the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well-known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, tonicity agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, sodium citrate, amino acids, sugars, polysorbate 80, etc. The concentration of immunoglobulin in these formulations can vary widely, *i.e.*, from less than about 0.1% to about 0.5%, usually at least about 1% to as much as about 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for injection could be made up to contain 1 ml sterile buffered water, and 1-100 mg of immunoglobulin, *e.g.*, about 5 or 10 mg. A typical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg to 1 g of immunoglobulin. Actual methods for preparing parentally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980), which is incorporated herein by reference.

The immunoglobulins of this invention can be frozen or lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immunoglobulins and art-known lyophilization and reconstitution techniques can be employed. It will be

appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees of immunoglobulin activity loss (*e.g.*, with conventional immunoglobulins, IgM antibodies tend to have greater activity loss than IgG antibodies) and that use levels may have to be adjusted to compensate.

The compositions containing the present humanized immunoglobulins or a cocktail thereof can be administered for therapeutic or prophylactic treatments. In therapeutic application, compositions are administered to a subject having a disease or disorder accompanied with angiogenesis including, for example, solid tumor, diabetic retinopathy, macular degeneration, Kaposi sarcoma, chronic articular rheumatism, psoriasis, angioma, scleroderma and neovascular glaucoma, in an amount sufficient to cure, inhibit, reduce or at least partially arrest the disease and its complications. The dosage ranges for the administration of antibodies of the invention are those large enough to produce the desired effect (*e.g.*, a reduction in angiogenic activity, tumor size etc.). The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of a VEGF-associated disorder or the desired change in the subject and can be determined by one of skill in the art. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's own immune system, but generally range from about 0.1 to 200 mg/kg of immunoglobulin per patient dose being commonly used. Specific dosing regimens with doses of 1 mg/kg, 5 mg/kg, 10 mg/kg, 20 mg/kg body weight, etc. administered daily, 2 or 3 per week, weekly, biweekly, monthly, etc. for a total of 1, 2, 4, 5-10 or more doses are all possible and would be selected by a skilled physician depending on the severity of the disease and other factors. It must be kept in mind that the materials of this invention may generally be

employed in serious disease states, that is, life-threatening or potentially life-threatening situations. In such cases, in view of the minimization of extraneous substances and the lower probability of "foreign substance" rejections which are achieved by the present humanized immunoglobulins of this invention, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these immunoglobulins. In addition, the immunoglobulins of the present invention may be administered in combination with other drugs including immunosuppresives and other anti-angiogenic factors such as anitsense molecules to VEGF. The subject can be any mammal, but is preferably a human.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of the immunoglobulin(s) of this invention sufficient to effectively treat the patient.

Diseases especially susceptible to therapy with the immunoglobulins of this invention (*e.g.*, humanized anti-VEGF antibody) include solid tumors believed to require angiogenesis or to be associated with elevated levels of VEGF, for example ovarian cancer, breast cancer, lung cancer (small cell or non-small cell), colon cancer, prostate cancer and brain tumors (*e.g.*, glioblastomas), In a one embodiment, the anti-VEGF antibody will be administered together with (*i.e.*, before, during or after) other anti-cancer therapy. For example, the anti-VEGF antibody may be administered together with any one or more of the chemotherapeutic drugs known to those of skill in the art of oncology, for example Taxol (paclitaxel) or its derivatives, platinum compounds such as carboplatin or cisplatin, anthracyclines such as doxorubicin, alkylating agents such as cyclophosphamide, anti-metabolites such as 5-fluorouracil, podophyllotoxins such as etoposide or any of the chemotherapeutic drugs or regimens described in Cancer: Principles & Practice of Oncology, 5th ed., Devita *et al.*, Lippincott-Raven Publishers, 1997 (which

is incorporated herein by reference for all purposes). The anti-VEGF antibody can be administered in combination with two, three or more of these agents in a standard chemotherapeutic regimen, for example taxol and carboplatin, for treating diseases such as breast and ovarian cancer. Other agents with which the anti-VEGF antibody can be administered include biologics such as monoclonal antibodies, including Herceptin™ against the HER2 antigen, or antibodies to the EGF receptor, or other anti-angiogenic drugs. Alternatively, the anti-VEGF antibody can be used together with radiation therapy or surgery.

Treatment (*e.g.*, standard chemotherapy) including the anti-VEGF antibody will increase the median progression-free survival or overall survival time of subjects with these tumors (*e.g.*, ovarian, breast, lung, and colon, especially when relapsed or refractory) when compared to the same treatment but without the anti-VEGF antibody. For example, using humanized antibodies derived from A.6.4.1 or MV833. it is expected that the median progression free or overall survival time will be increased by at least 30% or 40% but preferably 50%, 60% to 70% or even 100% or longer, compared to the same treatment (*e.g.*, chemotherapy) but without the anti-VEGF antibody. In addition or alternatively, treatment (*e.g.*, standard chemotherapy) including the anti-VEGF antibody will increase the complete response rate, partial response rate, or objective response rate (complete + partial) of subjects with these tumors (*e.g.*, ovarian, breast, lung, and colon, especially when relapsed or refractory) by at least 30% or 40% but preferably 50%, 60% to 70% or even 100% compared to the same treatment (*e.g.*, chemotherapy) but without the anti-VEGF antibody.

For example, treatment with the (*e.g.*, humanized) anti-VEGF antibody plus chemotherapy (*e.g.*, paclitaxel and carboplatin) will increase median progression-free survival in subjects with relapsed ovarian carcinoma by at least 40% or 50%, or by 75% or 100% or more (*e.g.*, from 9 months to 13 months or longer) relative to treatment with the same chemotherapy alone, and/or will increase the objective response rate by 40 or 50%, or by 75% or

100% or more (*e.g.*, from 30% or 40% to 50% or higher). Similarly, treatment with the anti-VEGF antibody plus chemotherapy (*e.g.*, ifosphamide, carboplatin and etoposide) will increase median progression-free survival in subjects with relapsed lung cancer (small cell or non-small cell) by at least 40% or 50%, or by 75% or 100% or more (*e.g.*, from 7 or 8 months to 11 or 12 months or longer) relative to treatment with the same chemotherapy alone, and/or will increase the objective response rate by 40% or 50%, or by 75% or 100% or more (*e.g.*, from 25% or 30% to 40% or 50% or higher).

Typically, in a clinical trial (*e.g.*, a phase II, phase II/III or phase III trial), the aforementioned increases in median progression-free survival and/or response rate of the subjects treated with chemotherapy plus the anti-VEGF antibody, relative to the control group of patients receiving chemotherapy alone (or plus placebo), will be statistically significant, for example at the $p = 0.05$ or 0.01 or even 0.001 level. It will also be understood by one of skill that the complete and partial response rates are determined by objective criteria commonly used in clinical trials for cancer, *e.g.*, as listed or accepted by the National Cancer Institute and/or Food and Drug Administration.

Diagnostics

In particular embodiments, compositions comprising humanized immunoglobulin of the present invention may be used to detect VEGF. Thus, a humanized immunoglobulin of the present invention, such as a humanized immunoglobulin that binds to the antigen determinant identified by the MV833 antibody may be labeled and used to identify anatomic sites that contain significant concentrations of VEGF. One or more labeling moieties may be attached to the humanized immunoglobulin including, but not limited to, radiopaque dyes, radiocontrast agents, fluorescent molecules, spin-labeled molecules, enzymes, or other labeling moieties of diagnostic value, particularly in radiologic or magnetic resonance imaging techniques.

For *in vivo* diagnostic imaging, the type of detection instrument available is a major factor in selecting a given radioisotope. The radioisotope chosen must have a type of decay, which is detectable for a given type of instrument. Still another important factor in selecting a radioisotope for *in vivo* diagnosis is that the half-life of the radioisotope be long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious radiation with respect to the host is minimized. Ideally, a radioisotope used for *in vivo* imaging will lack a particle emission, but produce a large number of photons in the 140-250 kev range, which may be readily detected by conventional gamma cameras.

For *in vivo* diagnosis, radioisotopes may be bound to immunoglobulin either directly or indirectly by using an intermediate functional group. Intermediate functional groups which often are used to bind radioisotopes which exist as metallic ions to immunoglobulins are the bifunctional chelating agents such as diethylenetriaminepentacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA) and similar molecules. Typical examples of metallic ions which can be bound to the antibodies of the invention are ^{111}In, ^{97}Ru, ^{67}Ga, ^{68}Ga, ^{72}As, ^{89}Zr, and ^{201}Tl.

The antibodies of the invention can also be labeled with a paramagnetic isotope for purposes of *in vivo* diagnosis, as in magnetic resonance imaging (MRI) or electron spin resonance (ESR). In general, any conventional method for visualizing diagnostic imaging can be utilized. Usually gamma and positron emitting radioisotopes are used for camera imaging and paramagnetic isotopes for MRI. Elements which are particularly useful in such techniques include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr, and ^{56}Fe.

The antibodies of the invention can be used to monitor the course of amelioration of a angiogenic- or VEGF-associated disorder. Thus, by measuring the increase or decrease of VEGF polypeptide present in various

body fluids or tissues, it would be possible to determine whether a particular therapeutic regiment aimed at ameliorating the disorder is effective.

Humanized immunoglobulins of the present invention can further find a wide variety of utilizes *in vitro*. By way of example, the immunoglobulins can be utilized for detection of VEGF polypeptides or fragments thereof.

For diagnostic purposes, the immunoglobulins may either be labeled or unlabeled. Unlabeled immunoglobulins can be used in combination with other labeled antibodies (second antibodies) that are reactive with the humanized immunoglobulin, such as antibodies specific for human immunoglobulin constant regions. Alternatively, the immunoglobulins can be directly labeled. A wide variety of labels may be employed, such as radionuclides, fluors, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, ligands (particularly haptens). Numerous types of immunoassays are available and are well known to those skilled in the art.

Kits can also be supplied for use with the immunoglobulins of the present invention. Therapeutic and diagnostic (*in vivo* and *in vitro*) kits are envisioned and may be used in the protection against or detection of a cellular activity or for the presence of a VEGF antigen. Thus, the immunoglobulin composition of the present invention may be provided, usually in a lyophilized form in a container, either alone or in conjunction with additional antibodies specific for the desired cell type. The immunoglobulins, which may be conjugated to a label or toxin, or unconjugated, are included in the kits and may include buffers (*e.g.*, Tris, phosphate, carbonate, etc.), as well as stabilizers, preservatives, biocides, inert proteins(*e.g.*, serum albumin, or the like), and a set of instructions for use. Generally, these materials will be present in less than about 5% wt. based on the amount of active immunoglobulin, and usually present in total amount of at least about 0.001% wt. Based again on the immunoglobulin concentration. Frequently, it will be desirable to include an inert extender or excipient to dilute the active ingredients, where the excipient

may be present in from about 1 to 99% wt. of the total composition. Where a second antibody capable of binding to the immunoglobulin is employed in an assay, this will usually be present in a separate vial. The second antibody is typically conjugated to a label and formulated in an analogous manner with the immunoglobulin formulations described above.

All publications mentioned herein are incorporated herein for all purposes. The following examples are offered by way of illustration, not by limitation. It will be understood that although the examples pertain to the humanized MV833 antibody, producing humanized antibodies with high binding affinity for the VEGF antigen it is also contemplated using CDR's from other monoclonal antibodies that bind to an epitope of VEGF.

EXAMPLES

Example 1: Preparation of a hybridoma producing the murine monoclonal antibody MV833

In order to obtain the mouse monoclonal antibody against VEGF, a hybridoma producing this antibody is prepared. First, a peptide to be used as an immunogen (antigen) is prepared. VEGF has the 121 amino acids shown in SEQ ID NO: 28. Either a peptide having the entire sequence or a peptide having a partial sequence thereof (*e.g.*, positions 21-30 or 97-102 of SEQ ID NO: 28) may be used.

These peptides may be obtained by conventional peptide synthesis (see, for example, Bodanszky, M and M.A. Ondetti, Peptide Synthesis, Interscience Publishers, New York (1966); Schroeder and Luebke, The Peptide, Academic Press, New York (1965); Nobuo Izumiya *et al.*, "Basics and Experiments of Peptide Synthesis", Maruzen Co., Ltd. (1975)).

The resultant VEGF or partial fragment thereof is dissolved in a buffer to prepare an antigen. If necessary, an adjuvant (*e.g.*, commercial Freund's complete or incomplete adjuvant) may be mixed therein to perform the immunization effectively.

The antigen is administered to a mammal, *e.g.*, a rodent such as rat, mouse and rabbit. The dose of the antigen is typically about 10-100μg per animal. The immunization is performed by injecting the antigen intravenously, subcutaneously or intraperitoneally. The interval between immunizations is not particularly limited; the interval may be several days to several weeks. Typically, the immunization is carried out at intervals of 2-3 weeks at least 2-3 times. After the final immunization, antibody-producing cells are harvested (*e.g.*, spleen cells, lymph node cells, peripheral blood cells and so forth). Among these, spleen cells are preferable.

In order to obtain a hybridoma, the antibody-producing cells are fused with myeloma cells. As the myeloma cell to be fused with the antibody-producing cell, a generally available, established cell line derived from an animal such as mouse may be used. A typical cell line to be used is one which has drug-selectivity and which cannot survive in HAT selection medium (containing hypoxanthine, aminopterin and thymidine) when unfused with the antibody-producing cell and can survive there only when fused with the cell. Specific examples of myeloma cells include mouse myeloma cell lines P3X63-Ag.8.U1 (P3U1), P3/NSI/1-Ag4-1 and Sp2/0-Ag14.

Subsequently, cell fusion of the above myeloma cell with the antibody producing cell is performed. The antibody producing cell and the myeloma cell are mixed at a ratio of 1:1-1:10 in an animal cell culture medium such as serum-free DMEM or RPMI-1640. In the presence of a cell fusion promoting agent such as polyethylene glycol or by electric pulse treatment (*e.g.*, electroporation), a fusion reaction is performed.

Hybridomas of interest are selected from the fused cells. Briefly, the cells are cultured in a medium containing hypoxanthine (10^{-3}-10^{-5} M), aminopterin (10^{-6}-10^{-7} M) and thymidine (10^{-5}-10^{-6} M), and those cells growing therein are obtained as hybridomas.

Subsequently the hybridomas are screened to determine whether an antibody of interest is present in the culture supernatants of the hybridomas. The screening of the hybridomas may be carried out by any conventional method. For example, an aliquot of culture supernatant from a tissue culture well where a hybridoma is growing can be collected and screened by enzyme immunoassay (EIA), radioimmunoassay (RIA), etc.

The cloning of the fused cell is performed, for example, by the dilution-culture method or the like to establish a hybridoma which is a cell producing the monoclonal antibody of interest.

As a method for recovering the monoclonal antibody from the established hybridoma, the conventional cell culture method or ascites formation method may be employed. In the cell culture method, the hybridoma is cultured in an animal cell culture medium such as 10% fetal bovine serum-containing RPMI-1640 medium, MEM medium or a serum-free medium for 2-10 days under usual culture conditions (*e.g.*, at 37 °C under 5% CO_2). The antibody is then obtained from the culture supernatant.

In the ascites formation method, the hybridoma (about $1x10^7$ cells) is administered into the abdominal cavity of an animal which is the same species as the animal from which the myeloma cell has been derived to thereby expand the hybridoma to a large quantity. One to two weeks thereafter, the ascites or serum is collected.

In the methods of recovering antibody described above, the antibody can be purified, if necessary, by appropriately selecting or combining conventional methods such as ammonium sulfate fractionation, ion exchange chromatography, affinity chromatography, gel chromatography and the like.

The hybridoma producing the mouse monoclonal antibody MV833 was deposited under the terms of the Budapest Treaty at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan)on September 24, 1996 under the Accession Number of FERM BP-5669.

Example 2: Cloning and sequencing of mouse MV833 variable region cDNAs

Mouse MV833 heavy and light chain variable region cDNAs were cloned from mRNA isolated from hybridoma cells using anchored PCR (Co *et al.*, J. Immunol. 148: 1149 (1992)). An outline of steps for cloning a cDNA coding for the mouse MV833 antibody variable region is shown in Fig. 1. The 5' primers used annealed to the poly-dG tails added to the cDNA, and the 3' primers to the constant regions. The amplified gene fragments were then inserted into the plasmid pUC18. Nucleotide sequences were determined from several independent clones for both V_L and V_H cDNA. For the heavy chain, a single, unique sequence was identified, typical of a mouse heavy chain variable region. For the light chain, two unique sequences, both homologous to murine light chain variable region sequences, were identified. However, one sequence was not functional because of a missing nucleotide that caused a frame shift at the V-J junction, and was identified as the non-productive allele. The other sequence was typical of a functional mouse kappa chain variable region. The variable region cDNA sequences of the heavy chain and the functional light chain and the translated amino acid sequences are shown in Figures 2 and 3.

The mouse V_K sequence belongs to Kabat's mouse kappa chain subgroup V. The sequence of the four N-terminal amino acids is somewhat unusual relative to other kappa chain sequences. However, that sequence is not unprecedented and has been reported before in the immunoglobulin database (Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed., U.S.

Department of Health Services, 1991). The mouse V_H belongs to Kabat's heavy chain subgroup II(A).

Example 3: Design of humanized MV833 variable regions

To retain the binding affinity of the mouse antibody in the humanized antibody, the general procedures of Queen *et al.* were followed (Queen *et al.* Proc. Natl. Acad. Sci. USA 86: 10029 (1989) and U.S. Patent Nos. 5,585,089 and 5,693,762). The choice of framework residues can be critical in retaining high binding affinity. In principle, a framework sequence from any human antibody can serve as the template for CDR grafting; however, it has been demonstrated that straight CDR replacement into such a framework can lead to significant loss of binding affinity to the antigen (Glaser *et al.*, J. Immunol. 149: 2606 (1992); Tempest *et al.*, Biotechnology 9: 266 (1992); Shalaby *et al.*, J. Exp. Med. 17: 217 (1992)). The more homologous a human antibody is to the original murine antibody, the less likely will the human framework introduce distortions into the mouse CDRs that could reduce affinity. Based on a sequence homology search against an antibody sequence database, the human antibody I2R was chosen as providing good framework homology to the mouse MV833 antibody. Other highly homologous human antibody chains would also be suitable to provide the humanized antibody framework, especially kappa light chains from human subgroup I and heavy chains from human subgroup I (as defined by Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th ed., U.S. Department of Health and Human Services, 1991).

The computer programs ABMOD and ENCAD (Levitt *et al.*, J. Mol. Biol. 168: 595 (1983)) were used to construct a molecular model of the MV833 variable domain, which was used to locate the amino acids in the MV833 framework that are close enough to the CDRs to potentially interact with them. To design the humanized MV833 heavy and light chain variable regions, the CDRs from the mouse MV833 antibody were grafted into the framework

regions of the human I2R antibody. At framework positions where the computer model suggested significant contact with the CDRs, the amino acids from the mouse antibody were substituted for the original human framework amino acids. For humanized MV833, this was done at residues 1, 27, 30, 37, 48 and 72 of the heavy chain and at residues 49 and 67 of the light chain. Furthermore, framework residues that occurred only rarely at their positions in the database of human antibodies were replaced by a human consensus amino acid at those positions. For humanized MV833 this was done at residues 16 and 115 of the heavy chain and at residue 43 of the light chain. A comparison of the framework regions of the murine MV833 and human I2R variable framework regions reveals that in the VL which has 80 framework amino acids, 54 amino acids are identical between the murine MV833 and the human I2R sequence. In other words there is 54/80 or 67.5% identity between the murine and human antibody amino acid sequences. (Fig. 16.) In the VH which has 84 framework amino acids, 61 are identical between the murine MV833 and the human I2R sequence. In other words there is 61/84 or 72.6% identity between the murine and human antibody amino acid sequences. (Fig. 17.)

The sequence of the humanized MV833 antibody heavy chain and light chain variable regions is shown in Figures 5 (SEQ ID NOs 5 and 6) and 6 (SEQ ID NOs 7 and 8). However, there are many potential CDR-contact residues that are also amenable to substitution and that may still allow the antibody to retain substantial affinity to the antigen. For example, the first four N-terminal amino acid residues in the humanized MV833 light chain can alternatively be substituted with the sequence from the murine antibody because of its contacts with the CDRs. The following Table 1 lists a number of positions in the framework where alternative amino acids may be suitable (LC = light chain, HC = heavy chain).

TABLE 1

Position	Humanized MV833	Alternatives
LC-1	D	E
LC-2	I	T
LC-3	Q	T
LC-4	M	V
LC-49	S	Y
LC-67	Y	S
HC-1	E	Q
HC-27	D	G, Y
HC-30	T	S
HC-37	M	V
HC-48	I	M
HC-72	S	A

Likewise, many of the framework residues not in contact with the CDRs in the humanized MV833 heavy and light chains can accommodate substitutions of amino acids from the corresponding positions of the human I2R antibody, from other human antibodies, from the mouse MV833 antibody, or from other mouse antibodies, without significant loss of the affinity or non-immunogenicity of the humanized antibody. The following Table 2 lists a number of additional positions in the framework where alternative amino acids may be suitable.

TABLE 2

Position	Humanized MV833	Alternatives
LC-43	A	P, V
HC-16	A	S
HC-115	T	I

Selection of various alternative amino acids may be used to produce versions of humanized MV833 that have varying combinations of affinity, specificity, non-immunogenicity, ease of manufacture, and other desirable properties. Thus, the examples in the above tables are offered by way of illustration, not of limitation.

Example 4: Construction of humanized MV833

Once the humanized variable region amino acid sequences had been designed as described above, genes were constructed to encode them, including signal peptides, splice donor signals and appropriate restriction sites (Figures 5 and 6). The light and heavy chain variable region genes were constructed and amplified using eight overlapping synthetic oligonucleotides ranging in length from approximately 60 to 80 bases (see He *et al.* J. Immunol. 160: 1029 (1998)). (Figs. 14 and 15). The oligos were annealed pairwise and extended with the Klenow fragment of DNA polymerase I, yielding four double-stranded fragments (Fig. 4; A to D). The resulting fragments were denatured, annealed, and extended with Klenow, yielding two fragments (Fig. 4; E and F). These fragments were denatured, annealed pairwise, and extended once again, yielding a full-length gene. The resulting product was amplified by polymerase chain reaction (PCR) using Taq polymerase, gel-purified, digested with XbaI, gel-purified again, and subcloned into the XbaI site of the pVk or pVg4 expression vector. The pVk vector for light chain expression has been previously described (see Co *et al.*, J. Immunol. 148:1149 (1992)). The pVg4

vector for heavy chain expression was constructed by replacing the XbaI - BamHI fragment of pVg1 containing the g1 constant region gene (see Co *et al.*, J. Immunol. 148: 1149 (1992)) with an approximately 2000 bp fragment of the human g4 constant region gene (Ellison and Hood, Proc. Natl. Acad. Sci. USA 79: 1984 (1982)) that extended from the HindIII site preceding the C_H1 exon of the g4 gene to 270 bp after the NsiI site following the C_H4 exon of the gene. Following are the specific reaction conditions utilized, however, these conditions and techniques are already well known and the particular conditions may be varied and still successful.

Cloning Ig V-genes by Anchored PCR

Purification of Total RNA

1. Wash 10 mls cells (5min., 1.1K, RT) once in 10 ml PBS. Decant and resuspend cells in residual buffer.

2. Add 1.2 ml RNA extraction buffer (50 mM NaOAc, pH 5.2; 1% SDS), vortex, incubate 2min. at RT. Transfer into 2 microfuge tubes.

3. Add 0.6 ml phenol (equilibrated twice in 1 M NaOAc, pH 5.2; twice in 50 mM NaOAc, pH 5.2), vortex, incubate 15min. at 65°, then 15min. on ice.

4. Spin (3min., 15K, RT), recover aqueous phase. Precipitate by addition of 0.1 vol 3 M NaOAc, pH 5.2 plus 2 vol absolute EtOH. Let stand 15min. on dry ice.

5. Spin (15min., 15K, 4°), resuspend in 2 x 0.2 ml 0.3 M NaOAc, pH 5.2 (combine 2 tubes into one). Spin briefly (3min., 15K, RT) to remove debris. Precipitate by addition of 2.5 vol (1 ml) absolute EtOH. Let stand 15min. on dry ice.

6. Spin (15min., 15K, 4°), air dry, resuspend pellet in 50 ml H_20. Measure OD_{260} of 1:250 dilution and calculate concentration (assuming 1 OD unit = 40 mg/ml RNA). Store unused portion at -20°, or re-precipitate (as in step 5) and store at -20° for long-term storage.

cDNA synthesis, dG tailing and PCR

1. Set up cDNA synthesis reaction as follows:

10 µg	RNA
4 µl	5x RT buffer (BRL)
2 µl	0.1 M DTT (BRL)
1 µl	20x dNTPs (10 mM ea; Pharmacia)
1 µl	RNasin (~ 40 U/ml; Promega)
2 µl	dT_{12-18} (20 mg/ml; Pharmacia)
2 µl	mMuLV-RT (200 U/ml; BRL)
	H_20 to 20 µl

Incubate 1h at 37°.

2. Add 180 µl TE, pH 7.5, extract once each with equal volumes phenol (buffer saturated; BRL), $CHCl_3$:isoamyl (24:1). Precipitate by addition of 0.1 vol (20 µl) 3 M NaOAc, pH 5.2 plus 2.5 vol. (500 µl) absolute EtOH. Let stand 15min. on dry ice. Spin (15min., 15K, 4°), wash w/ 200 µl chilled 70% EtOH, air dry, resuspend in 13 µl H_2O.

3. Set up dG tailing reaction as follows:

13 µl	cDNA
4 µl	5x TdT buffer (BRL)
2 µl	10 mM dGTP (Pharmacia)
1 µl	TdT (~15 U/ml; BRL)
20 µl	

Incubate 30min. at 37°.

4. Add 180 µl TE, pH 7.5, extract once each with equal volumes phenol (buffer saturated; BRL), $CHCl_3$:isoamyl (24:1). Precipitate by addition of 0.1 vol (20 ml) 3 M NaOAc, pH 5.2 plus 2.5 vol (500 µl) absolute EtOH. Let stand 15min. on dry ice. Spin (15min., 15K, 4°), wash w/ 200 µl chilled 70% EtOH, air dry, resuspend in 85 µl H_2O.

5. Set up 2 PCR reactions using micro PCR tubes (one for L, one for H) as follows:

20 µl	dG-cDNA
5 µl	MC045 (0.25 µg/µl)
5 µl	MC046 or MC047 (0.25 µg/µl)
6 µ	10x PCR buffer II (PE-Cetus)
5 µl	25 mM $MgCl_2$ (PE-Cetus)
1.2 µl	10 mM dNTPs (Pharmacia)
0.5 µl	AmpliTaq (5 U/ml; PE-Cetus)
17.3 µl	H_2O
60 µl	

Program PE9600 as follows (method 39):

2min. at 94°	(1 cycle)
5" at 95°, 5" at 55°, 30" at 72°	(35 cycles)
10min. at 72°	(1 cycle)
hold at 4°	

6. Load 3 x 20 µl samples on 0.8 % LMP agarose gel in 1x TBE, run 1-1.5 hours at 85V, excise bands under long-wave UV. Recover DNA using Sephaglas BandPrep Kit or phenol extraction method. Resuspend DNA in 10 µl TE, pH 7.5.

7. Digest with RI + H3 as follows:

10 µl	PCR product
2 µl	10x NEB2
2 µl	BSA (1:10)
1 µl	EcoRI (20 U/ml; NEB)
1 µl	HindIII (20 U/ml; NEB)
4 µl	H_2O
20 µl	

Incubate 2 hours at 37°. Digest 1 mg pUC19 in parallel.

8. Run reaction products on 0.8 % LMP agarose gel, and recover DNA using Sephaglas BandPrep Kit or phenol extraction method. Resuspend DNA in 11 µl TE, pH 7.5. Check 1 µl on minigel.

9. Ligate 2h-ON at 15°.

10. Transform 100 µl frozen competent DH1 by standard or rapid protocol.

Standard: 30min. on ice, 2min. at 37°, add 0.4 ml LB, inc 1h at 37°, plate 100 µl or 400 µl on LB-Amp plates.

Rapid: 40min. on ice, 2min. at 42°, 3min. on ice, plate 100 µl on LB-Amp plates.

11. Screen an appropriate number of colonies using Promega Wizard Prep Kit (or equivalent method). Confirm inserts with RI-H3 digest.

12. Prepare Qiagen MidiPrep DNA from 50 ml cultures grown in Terrific Broth (remember to add 1/10 vol K_2PO_4). Submit for sequencing analysis.

Anchored PCR V-gene Primers

5'-poly C (MC045)

Eco RI

5'-TATATCTAGAATTCCCCCCCCCCCCCCCC-3' (SEQ ID NO.25)

Xba I

3'-kappa (MC046)

Hind III

5'-TATAGAGCTCAAGCTTGGATGGTGGGAAGATGGATACAGTTGGTGC-3'

Sac I

(SEQ ID NO.26)

3'-heavy (MC047)

Hind III

5'- A G T

TATAGAGCTCAAGCTTCCAGTGGATAGACCGATGGGGCTGTCGTTTTGGC-

3' Sac I T

(SEQ ID NO.27)

Semi-Synthesis of Humanized Ig V-genes

Synthesis & Purification of Oligonucleotides

1. Synthesize 8 oligos per V-gene at 0.2 μmole scale, deprotect with NH_4OH, and lyophilize. Resuspend in 0.4 ml dH_20 and measure $OD_{260/280}$ of crude material. (Use the conversion $1OD_{260} = 33$ μg/ml)

2. Load 200 μg of each oligo in sequencing buffer on 10% polyacrylamide gel containing urea. Run gel until xylene cyanol dye has run ~2/3 of the way down the gel (4h at 400V).

3. Excise bands under long-wave UV illumination against a TLC plate. Recover DNA by crushing gel slices and soaking in extraction buffer (0.5 M NH_4OAc; 0.01 M MgOAc; 0.001 M EDTA; 0.1% SDS) ON at 37°.

4. Precipitate oligos by addition of 0.1 vol 3 M NaOAc, pH 5.2 plus 2.5 vol absolute EtOH. Let stand 15min. on dry ice. Spin (15min., 15K, 4°). Rinse with chilled 70% EtOH. Air dry. Resuspend in 20 μl dH_2O.

5. Measure $OD_{260/280}$ of purified oligos. Based on OD readings, check 1 μg each oligo on a 1% agarose minigel. Revise DNA concentrations as necessary based on minigel.

Assembly & PCR Amplification of V-genes

1. Combine oligos in pairwise fashion (4 reactions total, labeled A-D; see Fig.4) in micro PCR tubes as follows:

	A	B	C	D
1 µg 5' oligo	oligo 1	oligo 3	oligo 5	oligo 7
1 µg 3' oligo	oligo 2	oligo 4	oligo 6	oligo 8
10x Klenow buffer	2 µl	2 µl	2 µl	2 µl
H_2O	to 18 µl	to 18 µl	to 18 µl	to 18 µl

10'

Denature & renature using PCR machine: Program 21 = 5" at 95° ----> 5" at 25° (1 cycle).

Add:

dNTP (2 mM ea.)	1 µl	1 µl	1 µl	1 µl
Klenow (5U/µl)	1 µl	1 µl	1 µl	1 µl

Incubate 15min. at RT (room temperature).

2. Combine the following in micro PCR tubes (2 reactions total, labeled E-F; see Fig.4):

	E	F
reaction mixture	9 µl 'A'	9 µl 'C'
reaction mixture	9 µl 'B'	9 µl 'D'

Denature & renature using PCR machine (Program 21).

Add:

dNTP (2 mM ea.)	1 µl	1 µl
Klenow (5 U/µl)	1 µl	1 µl

Incubate 15min. at RT.

3. Combine the following in micro PCR tubes (1 reaction, labeled G; see Fig. 4):

	G
reaction mixture	10 µl 'E'
reaction mixture	10 µl 'F'
10x Klenow buffer	1 µl
H_2O	6.5 µl

Denature & renature using PCR machine (Program 21).

Add:

dNTP (2 mM ea.)	1.5 µl
Klenow (5 Uµl)	1 µl

Incubate 30min. at RT.

4. Set up the following PCR reaction in thin PCR tube:

5 µl 'G' reaction

2 µl 5' primer (0.25 µg/µl)
2 µl 3' primer (0.25 µg/µl)
10 µl 10x Taq extender buffer (Stratagene)
1 µl Taq Plus (5 U/µl; Stratagene)
70 µl H_2O
90 µl

Add 1 AmpliWax pellet (Perkin-Elmer). Incubate 2min. at 80°. Then overlay with 10 µl dNTP mix (2.5 mM ea.). Run Program 39 for 25 cycles:

2min. at 94° (1 cycle)
5" at 95°, 5" at 55°, 30" at 72° (25 cycles)
10min. at 72° (1 cycle)
hold at 4°

5. Purify PCR products on 0.8% LMP(Low melting point) agarose gel. Excise bands under long-wave UV and recover using Sephaglas BandPrep Kit or phenol extraction method. Resuspend in 20 µl elution buffer.

6. Digest purified PCR products with Xba I as follows:

20 µl PCR product
4 µl 10x NEB 2
4 µl BSA (1:10)
2 µl Xba I (20 U/ml)
10 µl H_2O
40 µl

Incubate 2h at 37°.

7. Purify on 0.8% LMP agarose gel. Excise bands under long-wave UV and recover using Sephaglas BandPrep Kit or phenol extraction method. Resuspend in 12 µl elution buffer. Check 1 µl on minigel.

8. Linearize appropriate expression vectors (*e.g.*, pVk & pVγ4) with Xba I (2h at 37°). Phosphatase with 1U CIP (Boehringer-Mannheim) per 2.5 µg vector. Purify on 0.8% LMP agarose gel. Excise bands under long-wave UV and recover DNA by extracting once each with phenol, phenol/$CHCl_3$ (1:1), and $CHCl_3$:isoamyl. Precipitate by addition of 0.1 vol 3M NaOAc pH 5.2 plus 2 vol absolute EtOH. Let stand 15min. on dry ice. Spin (15min., 15K, 4°). Resuspend in 12 µl elution buffer. Check 1 µl on minigel.

9. Ligate 2h-ON at 15°.

10. Transform 100 µl frozen competent DH1 with ligated DNA by standard or rapid protocol:

Standard: 30min. on ice, 2min. at 37°, add 0.4 ml LB, 1h at 37°, plate 100 or 400 µl on LB-Amp plates.

Rapid: 40min. on ice, 2min. at 42°, 3min. on ice, plate 100 µl on LB-Amp plates.

11. Screen by PCR:

2 µl 5' primer (0.25 µg/µl)
2 µl 3' primer (0.25 µg/µl)
5 µl 10x PCR buffer (Promega)
3 µl 25 mM $MgCl_2$ (Promega)

4 µl 2.5 mM dNTP (Pharmacia)
0.25 µl Taq (5U/µl; Promega)
34 µl H_2O
50 µl

Run Program 39 for 30 cycles. Analyze 20 µl PCR reaction product on an agarose gel alongside appropriate controls.

The structure of the final plasmids were verified by nucleotide sequencing and restriction mapping. All DNA manipulations were performed by standard methods well-known to those skilled in the art.

To construct a cell line producing humanized MV833, the heavy chain and light chain plasmids were transfected into the mouse myeloma cell line Sp2/0-Ag14 (ATCC CRL 1581). Before transfection, the heavy and light chain-containing plasmids were linearized using BstZ17I and FspI, respectively. Approximately 20 µg of each plasmid was transfected into 1×10^7 cells in PBS. Transfection was by electroporation using a Gene Pulser apparatus (BioRad) at 360 V and 25 µFD capacitance according to the manufacturer's instructions. The cells from each transfection were plated in four 96-well tissue culture plates, and after two days, selection medium (DMEM, 10% FCS, 1 x HT supplement (Sigma), 0.25 mg/ml xanthine, 1 µg/ml mycophenolic acid) was applied.

After approximately two weeks, the clones that appeared were screened for antibody production by ELISA. Antibody from a high-producing clone was prepared by growing the cells to confluency in regular medium (DMEM with 10% FCS), then replacing the medium with a serum-free medium (Hybridoma SMF; Gibco) and culturing until maximum antibody titers were achieved in the culture. The culture supernatant was run through a protein A-Sepharose column (Pharmacia); antibody was eluted with 0.1 M Glycine, 100 mM NaCl, pH 2.5 and subsequently exchanged into phosphate-buffered saline (PBS). The

purity of the antibody was verified by analyzing it on an acrylamide gel, and its concentration was determined by an OD_{280} reading, assuming 1.0 mg of antibody protein has an OD_{280} reading of 1.4.

Example 5: Properties of humanized MV833

The affinity of the murine and humanized MV833 antibody for VEGF was determined by competitive binding with radio-iodinated murine MV833 antibody. The procedure for the technique is described below:

1. Add 0.1 ml/tube $rhVEGF_{165}$ (R&D Systems, cat. # 293-VE/CF) at 50 ng/ml in 0.025 M carbonate-bicarbonate buffer, pH 9.0, 0.1 M NaCl, to Skatron Macrowell Tube Strips (part #15773). Incubate overnight at 4° C.

2. Decant and rinse tubes once with 0.3 ml/tube PBS-1% BSA. Add 0.3 ml/tube PBS-1% BSA, cover, and incubate 4 hr at 37° C.

3. Meanwhile, prepare 3-fold dilution series of murine MV833 or humanized MV833 in PBS-0.1% BSA ranging from 1.0 to 0.00001 µg/ml. Also prepare a solution containing (I-125)-murine MV833 at ~10 ng/ml (~1.0-1.5 x 10^5 cpm/ml) in PBS-0.1% BSA.

4. Decant and rinse tubes once with 0.3 ml/tube PBS-0.1% BSA. Add 0.1 ml/tube of each murine MV833 or humanized MV833 dilution followed by 0.1 ml (I-125)-murine MV833. Incubate overnight at 4° C.

5. Decant and wash tubes six times with 0.3 ml/tube PBS-0.1% BSA. Determine counts bound in a gamma counter.

The ratios of bound and free tracer antibody were then calculated and plotted. The binding affinities were calculated according to the methods of Berzofsky and Berkower (J. A. Berzofsky and I. J. Berkower, in Fundamental Immunology (ed. W.E. Paul), Raven Press (New York), 595 (1984)). The result shown in Figure 8 indicates that the affinity of the humanized MV833 is within 1.5-fold of the murine antibody (Kd of 1.8 $x10^{-10}$ M for humanized MV833 vs 1.2 x 10^{-10} M for murine MV833).

The purified humanized MV833 was subjected to isoelectric focusing (see Fig. 7). As a result, the isoelectric point was approximately PI 6.8. In Fig. 7, lane M represents an isoelectric point marker, and lane S represents a humanized MV833 antibody sample.

Example 6: VEGF neutralizing activity demonstrated on the vascular endothelial cell growth

Promotion Activity of VEGF

Human umbilical endothelial cells (2.5 x 10^3)(Sanko Pure Chemical) were cultured in a growth medium EGM-UV from the same manufacturer in Type I collagen-coated plates (Falcon). After trypsin treatment, the cells were recovered and dispensed into Type I collagen-coated 96-well plates (Iwaki Glass) (2.5 x 10^3 cells/well), followed by culturing for 24 hr. Then, the cells were washed twice with a medium for assaying neutralizing activity (45% D-MEM, 45% RPMI-1640, 10% FBS, 10 μg/ml bovine insulin, 5 μg/ml human transferrin, 10 nM sodium selenite, 10 μM 2-aminoethanol, 10μM 2-mercaptoethanol) and then cultured for 5 days in the same medium containing 50 ng/ml $VEGF_{165}$ (R&D) or 50 ng/ml $VEGF_{121}$ (S. Kondo *et al.*, Biochem. Biophys. Acta 1243:195 (1955)) and the humanized or murine MV833 at various concentrations. 1.85 KBq (925 Gbq/mmol)/well of ^{3}H-thymidine was added and, after 24 hr, treated with 0.25% trypsin-50 mM EDTA.

Subsequently, the cells were harvested on a glass filter using a cell harvester. The radioactivity was determined with a liquid scintillation counter.

As a result, the humanized MV833 (HU-MV833) exhibited VEGF neutralizing activity comparable to that of the mouse MV833 (Fig. 9).

Example 7: VEGF neutralization activity demonstrated on the receptor binding ability of VEGF

A culture supernatant expressing human VEGF receptor Flt-1 extracellular domain (2 μg/ml PBS) or human VEGF receptor KDR extracellular domain was added to Imuron 2 Strip (Dynatec) to coat and block the wells in the same manner as described in Example 5 above. 100,000 cpm/ml of ^{125}I-VEGF$_{165}$ (Amersham) with approximately 120 cpm/pg radioactivity was mixed with anti-VEGF antibody and left standing at room temperature for 1 hr. Then, 100 μl of this mixture was added to each of the wells and incubated for 3 hr. The wells were rinsed 3 times. The radioactivity was determined to calculate the amount of VEGF bound (Fig. 10). The experiment demonstrates that the humanized MV833 antibody of the invention has neutralizing activity comparable to that of the mouse MV833.

Example 8: Antitumor Activity

Human colon cancer cell line LS174T was transplanted subcutaneously into nude mice (BALB/c, nu/nu, 6-week old female) and subcultured. The subcultured tumor was cut out, and then a section thereof (2 mm x 2mm) was inoculated into the flank of each nude mouse.

One hundred μg/0.2 ml of the humanized or mouse MV833 antibody diluted with PBS was administered subcutaneously or intravenously every 4 days for 4 weeks from the day after the inoculation. The tumor size was then determined, followed by calculation of the tumor volume by the formula:

$(\text{Minor axis})^2$ x (Major axis)/2

As a result, the humanized antibody MV833 inhibited *in vivo* growth of the solid tumor caused by LS174T cells as effectively as the mouse MV833 (Fig. 11; ▲: Humanized MV833 was administered intravenously every 4 days,Δ: Humanized MV833 was administered subcutaneously every 4 days, ○: Mouse MV833 was administered intravenously every 4 days, and ●: Control).

Example 9: Crossreactivity with Mammal VEGFs

Macaque kidney-derived cell JTC-12 (RIKEN Gene Bank RCB0456), pig kidney-derived cell PK15 (RIKEN Gene Bank RCB0534), dog kidney-derived cell MDCK (RIKEN Gene Bank RCB0534), rabbit kidney-derived cell RK13 (Dainippon Pharmaceutical Co. No. 03-550), rat glioma C6 and mouse melanoma B16F1 were cultured separately in D-MEM. Also, guinea pig lung-derived cell JH4 (Dainippon Pharmaceutical Co. No. 08-158) was cultured in FM12K medium. The culture supernatant was recovered from each culture. The humanized MV833 antibody was labeled with biotin using an Amersham biotin labeling kit according to the manual attached to the kit. The reactivity of each culture supernatant was determined by sandwich EIA using the humanized MV833 bound to a solid phase as a primary antibody, 1000-fold dilution of the biotin-labeled humanized MV833 as a secondary antibody and 1000-fold dilution of POD-labeled streptavidin as a tertiary antibody. A positive reaction was observed in culture supernatants of monkey-, rabbit- and guinea pig-derived cells (Fig. 12; Panel A). This reactivity was inhibited by the addition of 5 μg/ml of the humanized MV833, but not inhibited by human IgG4 added in the same manner (Fig. 12; Panel B).

From these results, it was suggested that the humanized MV833 antibody binds to VEGFs from monkey, dog and guinea pig in addition to human VEGF. Further, the neutralizing ability of the humanized MV833

against the above animal-derived VEGFs was examined by Miles assay (A.A. Miles *et al.*, J. Physiol. (London), 118:228 (1952).

First, 0.5 ml of 0.1% Evans blue was injected intravenously into guinea pigs. Subsequently, 80 ng/ml of $VEGF_{165}$, a culture supernatant of monkey-derived cells (JTC-12), a culture supernatant of rabbit-derived cells (RK13), and a culture supernatant of guinea pig-derived cells (JH4) were prepared as test samples. In addition, a control group consisting of these samples alone, a group consisting of human IgG4 (80 μg/ml)-added samples, and a group consisting of the humanized MV833-added samples were prepared. Thirty minutes after the intravenous injection of Evans blue, a 0.1 ml sample of each of the above groups was administered to the guinea pigs subcutaneously. A determination was made on whether Evans blue appears subcutaneously in the treated animals or not to test subcutaneous permeability.

The results indicated that while the control group samples (culture supernatant alone) enhanced the subcutaneous permeability of Evans blue, the humanized MV833 inhibited the permeability (Fig. 13). In Fig. 13, at spotting points 1, 5 and 9, 80 ng/ml of $VEGF_{165}$ was administered. At spotting points 2, 6 and 10, a culture supernatant of monkey-derived cells (JTC-12) was administered. At spotting points 3, 7 and 11, a culture supernatant of rabbit-derived cells (RK13) was administered. At spotting points 4, 8 and 12, a culture supernatant of guinea pig-derived cells (JH4) was administered. "None" (1 -4) represents the control group. "hIgG4" (5- 8) represents the human IgG4-added group. "HuMV833" (9 -12) represents the human MV833-added group. The upper panel of Fig. 13 is a photograph showing the state of the skin in a guinea pig which was administered the samples subcutaneously. The lower panel shows the spotting points 1 to 12 in the subcutaneous administration.

Example 10: Examination of Anti-tumor Activity of MV833 and HuMV833

Anit-tumor activity of MV833 and HuMV833 was examined by measuring the inhibition of ascites formation in the presence of these anti-VEGF antibodies.

Human ovarian cancer cell line, K2 (obtained from Nagoya University School of Medicine, Nagoya, Japan),were cultured in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal calf serum at 37 °C in 5 % CO_2 under a humidified atmosphere. Cells were harvested using an EDTA solution (50 mM HEPES, 1 mM EDTA, 125 mM NaCl, 5 mM KCl, 5 mM glucose, pH 7.0) and suspended with PBS. Two hundred µl of cell suspension ($1.5x10^7$ cells) was inoculated i.p. into nude mice (BALB/c nu/nu, female, 5 years old)(day 0).

On day 4,8,12,16, and 20, 100µg of anti-VEGF monoclonal antibody, MV833 or HuM833, was administered i.v. through a lateral tail vein of mice (n=18 or 17). In a control group, mouse IgG instead of the anti-VEGF antibody was administered in the same manner (n=17). On day26, ascites fluids were removed following anesthetization with Nembutal to measure the volume of ascites fluid. The results are shown in Fig.18.

Female nude mice (n=17) in which 0.2ml of the cell suspension ($1.5x10^7$ cells) have been inoculated i.p. one hundred µg of anti-VEGF antibody, MV833 or HuMV833, was administered I.v. through a lateral tail vein of mice for every 4 days from the day after inoculation until the mice died and survival times of mice were obtained. The results are shown in Fig. 19.

As seen from Fig.18 and Fig.19, both the marked inhibition of ascites formation ($p<0.005$) and the prolongation of life spans ($p<0.01$ or $p<0.05$) were observed in the group in which each of the anti-VEGF antibodies was administered.

The inhibition of ascites re-accumulation by anti-VEGF antibodies was also examined.

From the mice in which ascites fluids were observed 23 days after the tumor cell inoculation, the ascites fluids were removed by aspiration (day 0). The mice were randomly divided into three groups: two anti-VEGF antibody-administering groups (n=15) and control group (n=15).

On day 0 and 4, 100μg of anti-VEGF antibody or mouse IgG was administered i.v. through a lateral tail vein of the mice of each group. On day 5, the re-accumulated ascites fluids were removed from all of the individuals, to measure the volume of the ascites. The results are shown in Fig. 20.

Female nude mice (n=16 or 17), from which ascites fluids were removed 23 days after the inoculation, were administered with 100ug of anti-VEGF antibody or mouse IgG for every 4 days from the day of removal of ascites until the mice died. Survival days were obtained in the mice. The results are shown in Fig. 21.

As seen from Fig.20 and Fig.21, both the marked inhibition of ascites re-accumulation ($p<0.005$) and the prolongation of life spans ($p<0.01$ or $p<0.05$) were observed in the murine group in which each of the anti-VEGF antibodies was administered.

In addition, an examination for the antitumor activity of anti-VEGF monoclonal antibody combined with taxol was also conducted. Human fibrosarcoma (HT-1080) tumors, which has been taken from maintenance nude mice, cut into 2 x 2 x 2 mm pieces and transplanted into the abdominal region of native nude mice (6 mice/group) using trocar(day 0). Taxol (10mg/kg) was administered i.v. through tail vein of mice on day 1 and 4. The anti-VEGF antibody (12.5 μg/mouse) was administered i.v. through tail vein of mice on day 1,5,9,13,17. The diameter of the tumor was measured over time, whereby the volume of the tumor was calculated.

The results are shown in Fig.22 and Fig.23. As seen in these figures, the group administered with the combination of the antibody and taxol showed a

higher anti-tumor activity than the groups administered with the antibody alone or with the taxol alone.

What is claimed is

1. A humanized antibody that specifically binds to Vascular Endothelial Cell Growth Factor (VEGF).

2. The humanized antibody of claim 1, wherein the humanized antibody is derived from the murine MV833 antibody.

3. The humanized antibody of claim 1, wherein the humanized antibody contains a CDR from a sequence as set forth in SEQ ID NO:2.

4. The humanized antibody of claim 1, wherein the humanized antibody comprises complementarity determining regions from the mouse MV833 antibody and heavy and light chain variable region frameworks from human I2R antibody heavy and light chain frameworks, provided that at least one position selected from the group consisting of LC49, LC67, HC1, HC27, HC30, HC37, HC48,and HC72 is occupied by the amino acid present in the equivalent position of the mouse MV833 antibody heavy or light chain variable region framework, which humanized antibody specifically binds to VEGF with an affinity constant between $10^7\ M^{-1}$ and ten-fold the affinity of the mouse VEGF antibody.

5. The humanized antibody of claim 4, provided that each position selected from the group consisting of LC49, LC67, HC1, HC27, HC30, HC37, HC48,and HC72 is occupied by the amino acid present in the equivalent position of the mouse MV833 antibody heavy or light chain variable region framework.

6. The humanized antibody of claim 5, provided that at least one position selected from the HC16, HC115, and LC43 is occupied by an amino acid present in the equivalent position of a human antibody heavy or light chain consensus sequence.

7. The humanized antibody of claim 1, comprising a heavy chain variable region sequence as set forth in SEQ. ID. NO:8 starting at position 1 and a light chain variable region sequence as set forth in SEQ. ID. NO:6 starting at position 1.

8. The humanized antibody of claim 7, wherein in one or more positions selected from the group consisting of LC1, LC2, LC3, LC4, LC43, LC49, LC67, HC1, HC16, HC27, HC30, HC37, HC48, HC72, and HC115 may be substituted as shown in Tables 1 and 2.

9. The humanized antibody of claim 1, comprising a humanized heavy chain having at least 85% identity with the humanized heavy chain as set forth in SEQ. ID. NO:8 and a humanized light chain having at least 85% sequence identity with the humanized light chain as set forth in SEQ. ID. NO:6, provided that at least one position selected from the group consisting of LC1, LC2, LC3, LC4, LC43, HC16, HC116 is occupied by the amino acid present in the equivalent position of the mouse MV833 antibody heavy or light chain variable region framework.

10. The humanized antibody of claim 1, wherein the antibody comprises two pairs of light/heavy chain dimmers, wherein each chain comprises a variable region and a constant region.

11. The humanized antibody of claim 1, wherein the antibody comprises an Fab or $F(ab')_2$ fragment.

12. The humanized antibody of claim 1, wherein the antibody is an IgG_1 immunoglobulin subtype.

13. The humanized antibody of claim 1, wherein the antibody is in purified form.

14. The humanized antibody of claim 1, wherein the antibody is in lyophilized form.

15. The humanized antibody of claim 1, wherein the constant region is a Cγ4 region.

16. The humanized antibody of claim 1, wherein the antibody competes with mouse antibody MV833 for specific binding to VEGF.

17. A polynucleotide sequence encoding the humanized antibody of claim 1.

18. The polynucleotide of claim 17, wherein the light chain of the humanized antibody is encoded by a sequence as set forth in SEQ ID NO:1 or SEQ ID NO:5.

19. The polynucleotide of claim 17, wherein the heavy chain of the humanized antibody is encoded by a sequence as set forth in SEQ ID NO:3 or SEQ ID NO:7.

20. The polynucleotide of claim 17, having a sequence as set forth in SEQ ID Nos: 5 and 7.

21. A vector containing the polynucleotide sequence of claim 17.

22. The vector of claim 21, wherein the vector is an expression vector.

22. A host cell containing the vector of claim 21.

23. The host cell of claim 22, wherein the host cell is a bacterial cell.24.

24. The host cell of claim 22, wherein the host cell is a mammalian cell.

25. A method of producing humanized VEGF antibody, comprising:culturing the host cell of claim 22, under conditions whereby the humanized antibody is expressed; and recovering the humanized antibody.

26. A pharmaceutical composition comprising the humanized antibody of claim 1 and a pharmaceutically acceptable carrier.

27. A method of inhibiting an angiogenesis-associated disorder in subject comprising administering to the subject an effective dosage of a human or humanized antibody that specifically binds to VEGF.

28. The method of claim 27, wherein the angiogenesis-associated disorder is a VEGF-associated disorder.

29. The method of claim 27, wherein the angiogenesis-associated disorder is selected from the group consisting of a cancer, solid tumor, diabetic retinopathy, macular degeneration, Kaposi sarcoma, chronic articular rheumatism, psoriasis, angioma, scleroderma and/or neovascular glaucoma.

30. The method of claim 29, wherein the cancer is selected from the group consisting of a breast cancer, an ovarian cancer, a lung cancer and a colon cancer.

31. The method of claim 27, wherein the subject is a mammal.

32. The method of claim 28, wherein the mammal is a human.

33. The method of claim 27, wherein the humanized antibody is derived from the murine MV833 antibody.

34. The method of claim 27, wherein the humanized antibody contains a CDR from a sequence as set forth in SEQ ID NO:2.

35. The method of claim 27, wherein the humanized antibody comprises complementarity determining regions from the mouse MV833 antibody and heavy and light chain variable region frameworks from human I2R antibody heavy and light chain frameworks, provided that at least one position selected from the group consisting of LC49, LC67, HC1, HC27, HC30, HC37, HC48, and HC72 is occupied by the amino acid present in the equivalent position of the mouse MV833 antibody heavy or light chain variable region framework, which humanized antibody specifically binds to VEGF with an affinity constant between $10^7\ M^{-1}$ and ten-fold the affinity of the mouse VEGF antibody.

36. The method of claim 35, wherein each position selected from the group consisting of LC49, LC67, HC1, HC27, HC30, HC37, HC48, and HC72 is

occupied by the amino acid present in the equivalent position of the mouse MV833 antibody heavy or light chain variable region framework.

37. The method of claim 36, wherein at least one position selected from the HC16, HC115, and LC43 is occupied by an amino acid present in the equivalent position of a human antibody heavy or light chain consensus sequence.

38. The method of claim 27, wherein the humanized antibody comprises a heavy chain variable region sequence as set forth in SEQ. ID. NO:8 and a light chain variable region sequence as set forth in SEQ. ID. NO:6.

39. The method of claim 38, wherein in one or more positions selected from the group consisting of LC1, LC2, LC3, LC4, LC43, LC49, LC67, HC1, HC16, HC27, HC30, HC37, HC48, HC72, and HC115 may be substituted as shown in Tables 1 and 2.

40. The method of claim 27, wherein the humanized antibody comprises a humanized heavy chain having at least 85% identity with the humanized heavy chain as set forth in SEQ. ID. NO:8 and a humanized light chain having at least 85% sequence identity with the humanized light chain as set forth in SEQ. ID. NO:6, provided that at least one position selected from the group consisting of LC1, LC2, LC3, LC4, LC43, HC16, HC116 is occupied by the amino acid present in the equivalent position of the mouse MV833 antibody heavy or light chain variable region framework.

41. The method of claim 27, wherein the antibody comprises two pairs of light/heavy chain dimmers, wherein each chain comprises a variable region and a constant region.

42. The method of claim 27, wherein the antibody comprises an Fab or $F(ab')_2$ fragment.

43. A method of increasing the median progression-free survival time of a subject having a cancer, comprising administering to the subject a standard chemotherapeutic regimen and a humanized antibody that binds to VEGF, wherein the median progression-free survival time is increased compared to a subject administered the chemotherapeutic regimen without the humanized antibody.

44. The method of claim 43, wherein the subject is a mammal.

45. The method of claim 44, wherein the mammal is a human.

46. The method of claim 43, wherein the cancer is selected from the group consisting of a breast cancer, an ovarian cancer, a lung cancer, and a colon cancer.

47. The method of claim 43, wherein the humanized antibody neutralizes VEGF.

48. The method of claim 43, wherein the increase is by at least 50%.

49. A method of increasing the objective response rate of a subject having a cancer comprising administering to the subject a standard chemotherapeutic regimen and a humanized antibody that binds to VEGF, wherein the objective response rate is increased compared to a subject treated with the standard chemotherapeutic regimen without the humanized antibody.

50. The method of claim 49, wherein the subject is a mammal.

51. The method of claim 50, wherein the mammal is a human.

52. The method of claim 49, wherein the cancer is selected from the group consisting of a breast cancer, an ovarian cancer, a lung cancer and a colon cancer.

53. The method of claim 49, wherein the humanized antibody neutralizes VEGF.

54. The method of claim 49, wherein the increase is by at least 50%.

55. The method of diagnosing an angiogenesis-associated disorder, comprising contacting a sample with a humanized antibody that binds to VEGF;

detecting binding of the antibody; and

comparing the amount of binding to a control sample, wherein an increase in binding is indicative of an angiogenesis-associated disorder.

56. The method of claim 55, wherein the sample is a tissue sample.

57. The method of claim 55, wherein the angiogenesis-associated disorder is selected from the group consisting of a cancer, solid tumor, diabetic retinopathy, macular degeneration, Kaposi sarcoma, chronic articular rheumatism, psoriasis, angioma, scleroderma and/or neovascular glaucoma.

58. The method of claim 57, wherein the cancer is selected from the group consisting of a breast cancer, an ovarian cancer, a lung cancer and a colon cancer.

59. A method for determining the prognosis of a subject having an angiogenesis-associated disorder comprising:

a) contacting the subject with a humanized antibody the binds to VEGF;

b) determining the level of VEGF polypeptide in the subject; and

b) correlating the level with prognosis of the subject.

60. The method of claim 59, further comprising monitoring the subject for recovery from the angiogenesis effects of the angiogenesis-associated disorder.

FIG.1

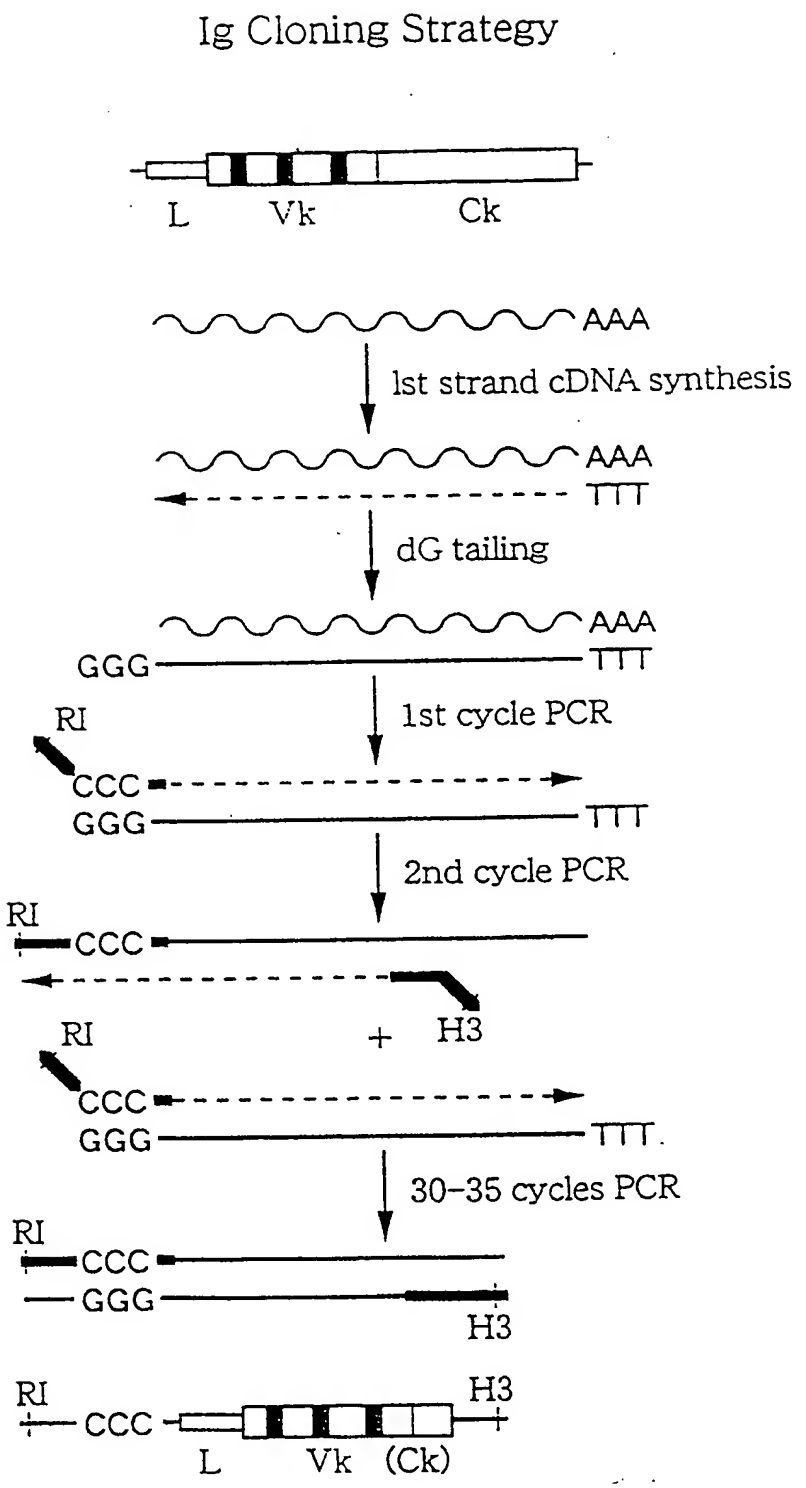

Ig Cloning Strategy
L
Vk
Ck
AAA
1st strand cDNA synthesis
AAA
TTT
dG tailing
AAA
GGG
TTT
RI
1st cycle PCR
CCC
GGG
TTT
2nd cycle PCR
RI
CCC
RI
+
H3
CCC
GGG
TTT.
30-35 cycles PCR
RI
CCC
GGG
H3
RI
H3
CCC
L
Vk
(Ck)

FIG. 2

Mouse MV833 light chain variable region sequence

```
                                                30                                                  60
ATG ACC ATG TTC TCA CTA GCT CTT CTC CTC AGT CTT CTG CTC CTC TGT GTC TCC GAT TCT
 M   T   M   F   S   L   A   L   L   L   S   L   L   L   L   C   V   S   D   S
                                                90                                                 120
AGG GCA GAA ACA ACT GTG ACC CAG TCT CCA GCA TCC CTG TCC ATG GCT ATA GGA GAA AAA
 R   A   E   T   T   V   T   Q   S   P   A   S   L   S   M   A   I   G   E   K
                                               150                                                 180
GTC ACC ATC AGA TGC ATA ACC AGC AAT GAT ATT GAT GAT GAT ATG AAC TGG TAC CAG CAG
 V   T   I   R   C   I   T   S   N   D   I   D   D   D   M   N   W   Y   Q   Q
                                               210                                                 240
AAG CCA GGG GAA CCT CCT AAG CTC CTT ATT TCA GAA GGC AAT ACT CTT CGT CCT GGA GTC
 K   P   G   E   P   P   K   L   L   I   S   E   G   N   T   L   R   P   G   V
                                               270                                                 300
CCA TCC CGA TTC TCC AGC AGT GGC TAT GGT ACA GAT TTT GTT TTT ACA ATT GAA GAC ATA
 P   S   R   F   S   S   S   G   Y   G   T   D   F   V   F   T   I   E   D   I
                                               330                                                 360
CTC TCA GAA GAT GTT GCA GAT TAC TAC TGT TTC CAA AGT GAT AAC TTG CCG TAC ACG TTC
 L   S   E   D   V   A   D   Y   Y   C   F   Q   S   D   N   L   P   Y   T   F

GGC GGG GGG ACC AAG CTG GAA ATA AAA
 G   G   G   T   K   L   E   I   K
```

FIG. 3

Mouse MV833 heavy chain variable region sequence

```
                                                30                                                  60
ATG GAA TGG AGT TGG ATA TTT CTC TTT CTC CTG TCA GGA ACT GCA GGT GTC CAC TCT GAG
 M   E   W   S   W   I   F   L   F   L   L   S   G   T   A   G   V   H   S   E
                                                90                                                 120
GTC CAA CTG CAG CAG TCT GGA CCT GAG CTG GTA AAG CCT GGG GCT TCA GTG AAG ATG TCC
 V   Q   L   Q   Q   S   G   P   E   L   V   K   P   G   A   S   V   K   M   S
                                               150                                                 180
TGC AAG GCT TCT GGA GAC ACA TTC ACT ACC TAT GTT ATA CAC TGG ATG AAG CAG AAG CCT
 C   K   A   S   G   D   T   F   T   T   Y   V   I   H   W   M   K   Q   K   P
                                               210                                                 240
GGG CAG GGC CTT GAG TGG ATT GGA TAT ATT AAT CCT TAC AAT GAT GGT ACT AAG TAC AAT
 G   Q   G   L   E   W   I   G   Y   I   N   P   Y   N   D   G   T   K   Y   N
                                               270                                                 300
GAG AAG TTC AAA GGC AAG GCC ACA CTG ACT TCA GAC AAA TCC TCC AGC ACA GCC TAC ATG
 E   K   F   K   G   K   A   T   L   T   S   D   K   S   S   S   T   A   Y   M
                                               330                                                 360
GAG CTC AGC AGC CTG ACC TCT GAG GAC TCT GCG GTC TAT TAC TGT GCA AGG ATC TAC TAT
 E   L   S   S   L   T   S   E   D   S   A   V   Y   Y   C   A   R   I   Y   Y
                                               390
GAT TAC GAC GGG GAC TAC TGG GGC CAA GGC ACC ACT CTC ACA GTC TCC TCA
 D   Y   D   G   D   Y   W   G   Q   G   T   T   L   T   V   S   S
```

FIG. 4

Strategy for Semi-Synthesis of Humanized Ig V-genes

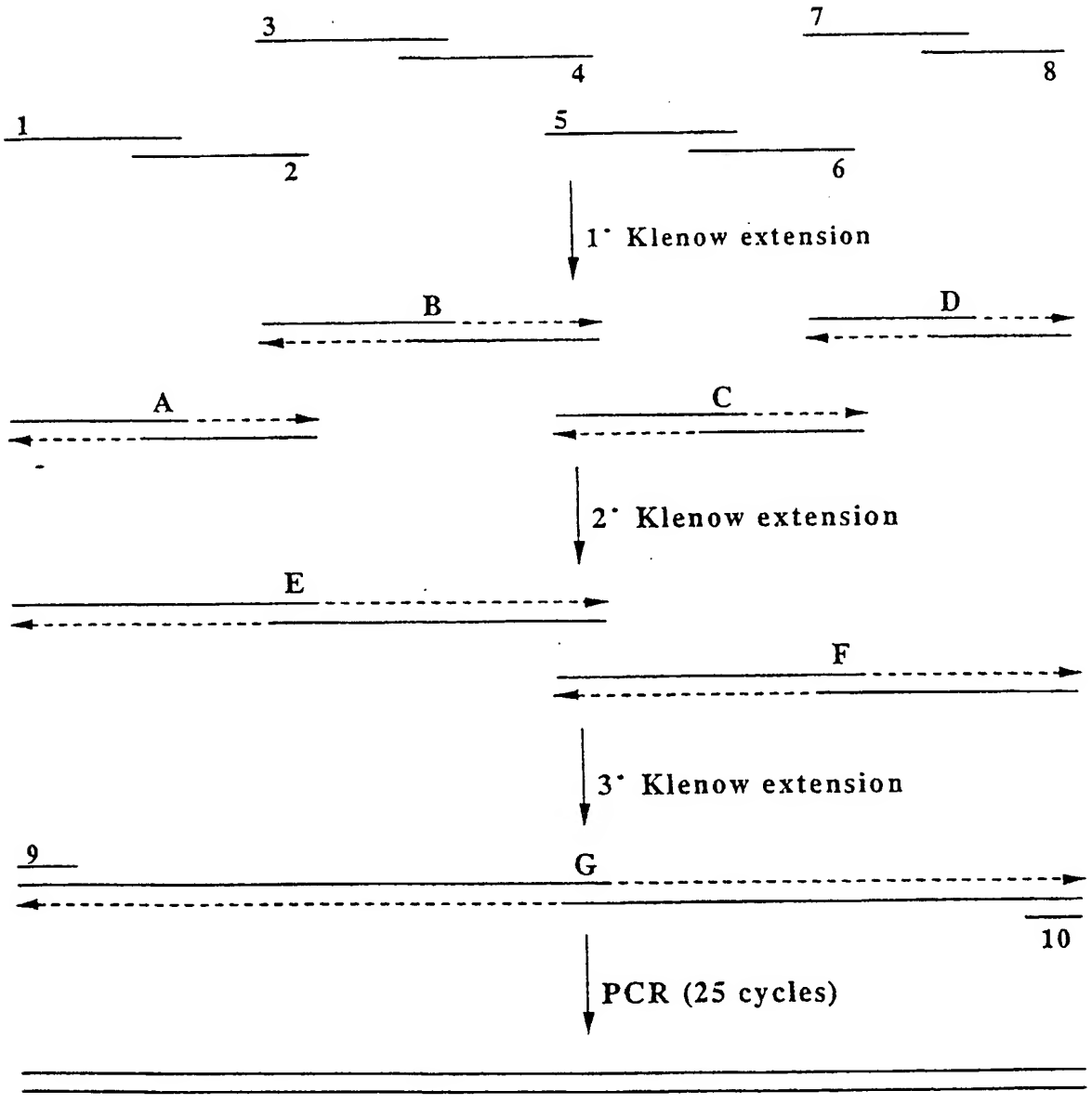


FIG. 5

Humanized MV833 light chain variable region sequence

```
                                                30                                                  60
ATG ACC ATG TTC TCA CTA GCT CTT CTC CTC AGT CTT CTG CTC CTC TGT GTC TCC GAT TCT
 M   T   M   F   S   L   A   L   L   L   S   L   L   L   L   C   V   S   D   S
                                                90                                                 120
AGG GCA GAT ATC CAG ATG ACC CAG TCT CCG AGC TCC CTG TCC GCA TCT GTG GGA GAC AGA
 R   A   D   I   Q   M   T   Q   S   P   S   S   L   S   A   S   V   G   D   R
                                               150                                                 180
GTC ACC ATC ACT TGC ATA ACC AGC AAT GAT ATT GAT GAT GAT ATG AAC TGG TAT CAG CAG
 V   T   I   T   C   I   T   S   N   D   I   D   D   D   M   N   W   Y   Q   Q
                                               210                                                 240
AAG CCA GGG AAA GCT CCT AAG CTT CTT ATT TCA GAA GGC AAT ACT CTT CGT CCT GGA GTC
 K   P   G   K   A   P   K   L   L   I   S   E   G   N   T   L   R   P   G   V
                                               270                                                 300
CCA TCC CGA TTC TCC GGA AGT GGC TAT GGT ACA GAT TTT ACT CTC ACA ATT AGC AGC CTG
 P   S   R   F   S   G   S   G   Y   G   T   D   F   T   L   T   I   S   S   L
                                               330                                                 360
CAG CCT GAA GAT GTT GCA ACT TAC TAC TGT TTC CAA AGT GAT AAC TTG CCG TAC ACG TTT
 Q   P   E   D   V   A   T   Y   Y   C   F   Q   S   D   N   L   P   Y   T   F
                                               390
GGC CAA GGG ACC AAG GTG GAA ATA AAA
 G   Q   G   T   K   V   E   I   K
```

FIG. 6

Humanized MV833 heavy chain variable region sequence

```
                                        30                                  60
ATG GAA TGG AGT TGG ATA TTT CTC TTT CTC CTG TCA GGA ACT GCA GGT GTC CAC TCT GAG
 M   E   W   S   W   I   F   L   F   L   L   S   G   T   A   G   V   H   S   E
                                        90                                 120
GTC CAA CTG GTG CAG TCT GGA GCT GAG GTG AAG AAG CCT GGA GCT TCA GTG AAG GTG TCC
 V   Q   L   V   Q   S   G   A   E   V   K   K   P   G   A   S   V   K   V   S
                                       150                                 180
TGC AAA GCT TCT GGA GAC ACA TTC ACT ACC TAT GTT ATA CAC TGG ATG CGT CAG GCT CCT
 C   K   A   S   G   D   T   F   T   T   Y   V   I   H   W   M   R   Q   A   P
                                       210                                 240
GGG CAG GGT CTC GAG TGG ATT GGA TAT ATT AAT CCT TAC AAT GAT GGT ACT AAG TAC AAT
 G   Q   G   L   E   W   I   G   Y   I   N   P   Y   N   D   G   T   K   Y   N
                                       270                                 300
GAG AAG TTC AAA GGC AGA GTC ACA ATC ACT TCA GAC AAA TCC ACC AGC ACA GCC TAC ATG
 E   K   F   K   G   R   V   T   I   T   S   D   K   S   T   S   T   A   Y   M
                                       330                                 360
GAG CTC AGC AGC CTG AGG TCT GAG GAC ACT GCG GTC TAT TAC TGT GCA AGG ATC TAC TAT
 E   L   S   S   L   R   S   E   D   T   A   V   Y   Y   C   A   R   I   Y   Y
                                       390                                 420
GAT TAC GAC GGA GAC TAC TGG GGT CAA GGC ACC CTG GTC ACA GTC TCC TCA
 D   Y   D   G   D   Y   W   G   Q   G   T   L   V   T   V   S   S
```

FIG. 7

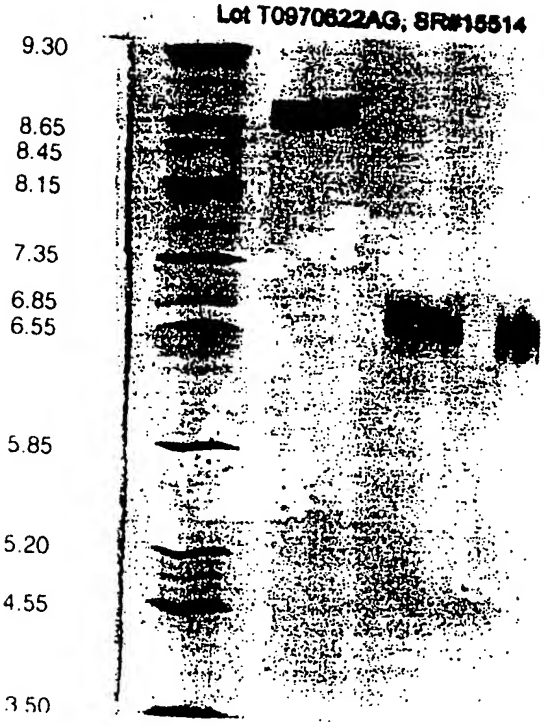

M
S
Lot T0970622AG; SR#15514
9.30
8.65
8.45
8.15
7.35
6.85
6.55
5.85
5.20
4.55
3.50

FIG. 8

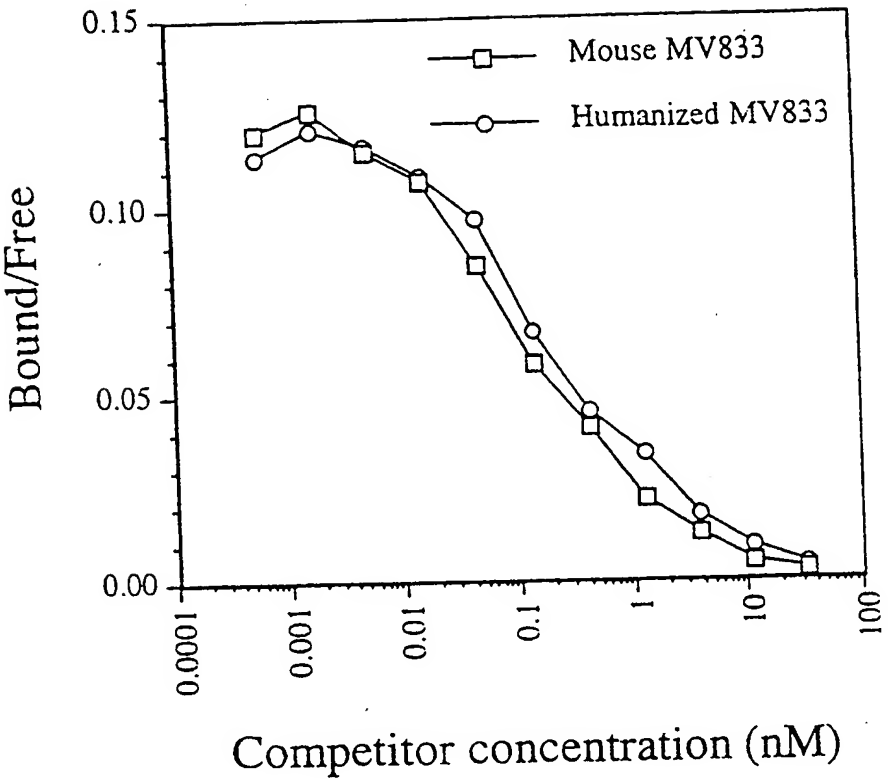

0.15
0.10
0.05
0.00
Bound/Free
Mouse MV833
Humanized MV833
0.0001
0.001
0.01
0.1
1
10
100
Competitor concentration (nM)

FIG. 9 A

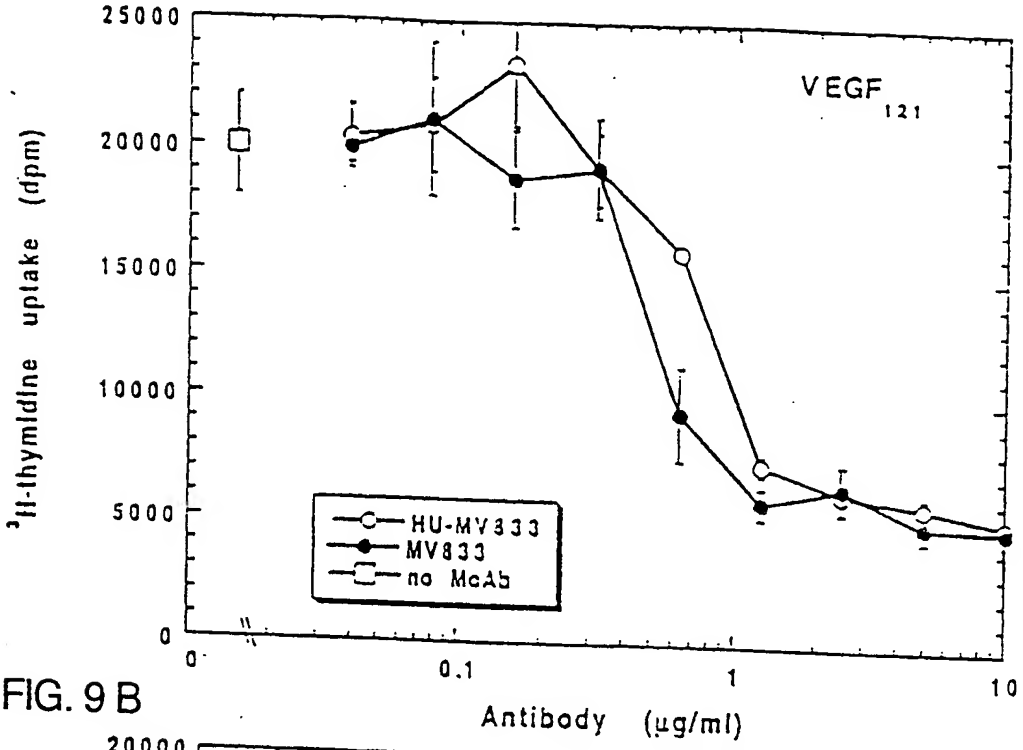

VEGF 121
^{3}H-thymidine uptake (dpm)
25000
20000
15000
10000
5000
0
0
0.1
1
10
Antibody (μg/ml)
HU-MV833
MV833
no MoAb

FIG. 9 B

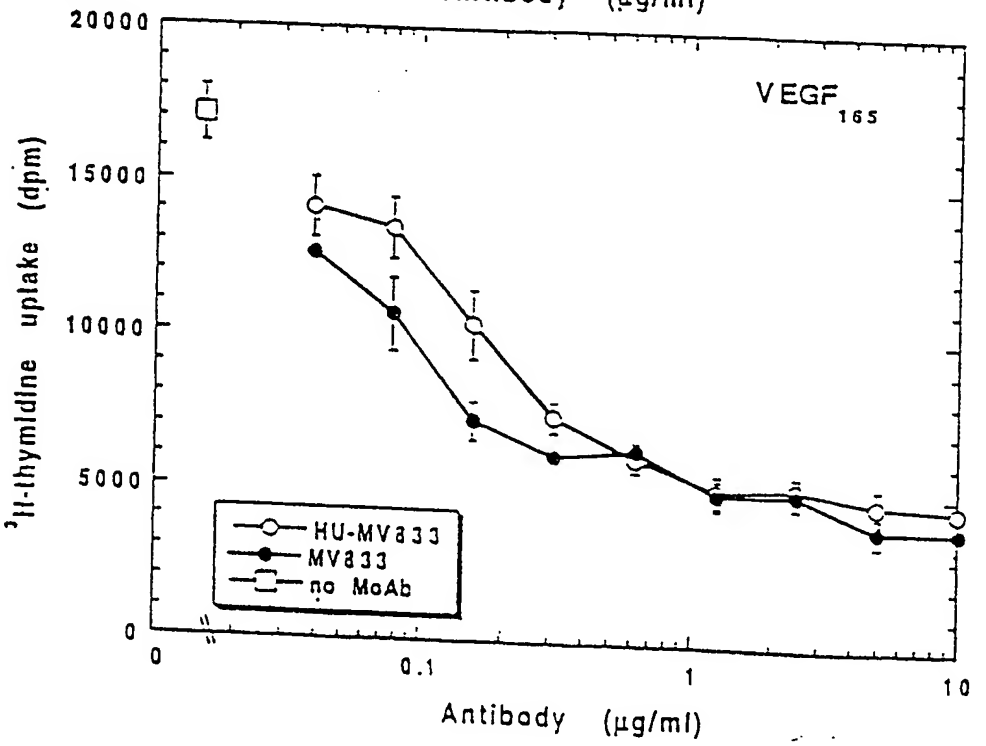

VEGF 165
^{3}H-thymidine uptake (dpm)
20000
15000
10000
5000
0
0
0.1
1
10
Antibody (μg/ml)
HU-MV833
MV833
no MoAb

FIG. 10 A

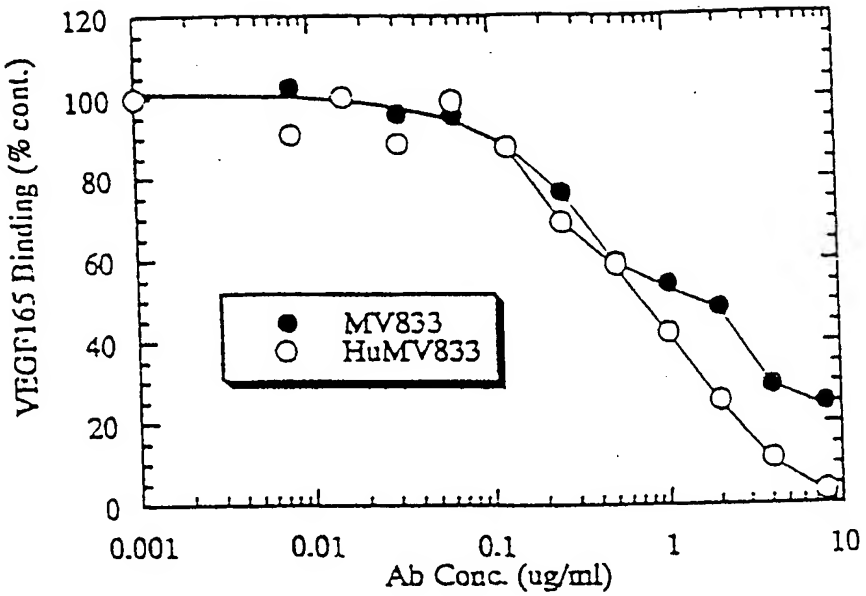


FIG. 10 B

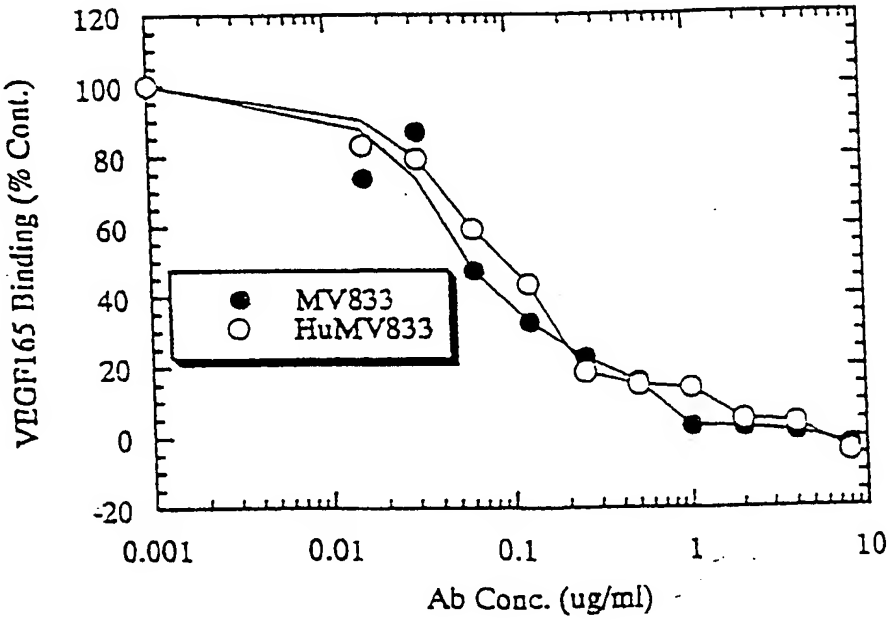


FIG. 11

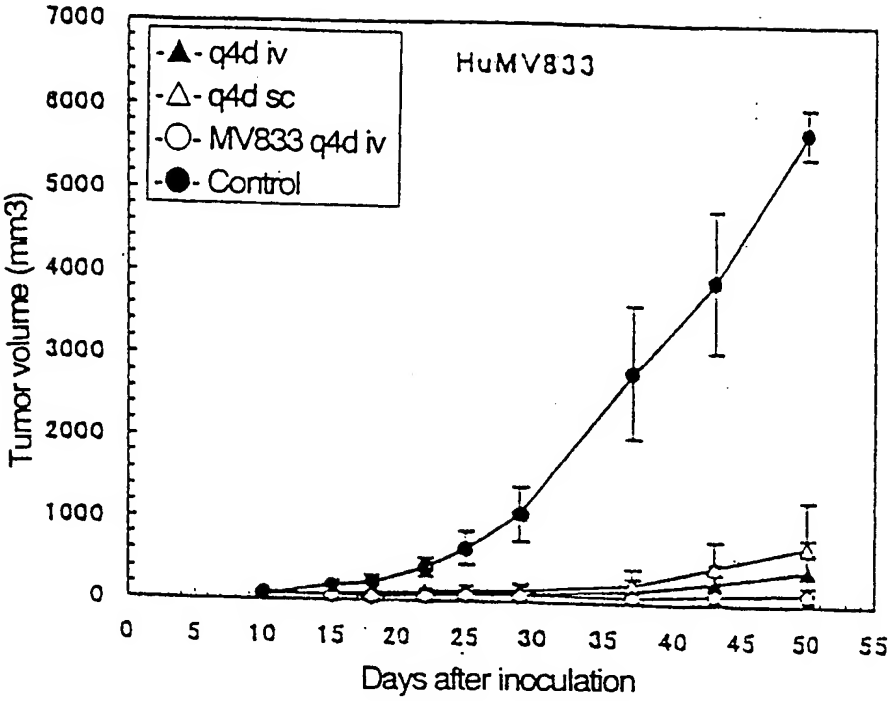

HuMV833
-▲- q4d iv
-△- q4d sc
-○- MV833 q4d iv
-●- Control
Tumor volume (mm3)
7000
6000
5000
4000
3000
2000
1000
0
0 5 10 15 20 25 30 35 40 45 50 55
Days after inoculation

FIG. 12 A

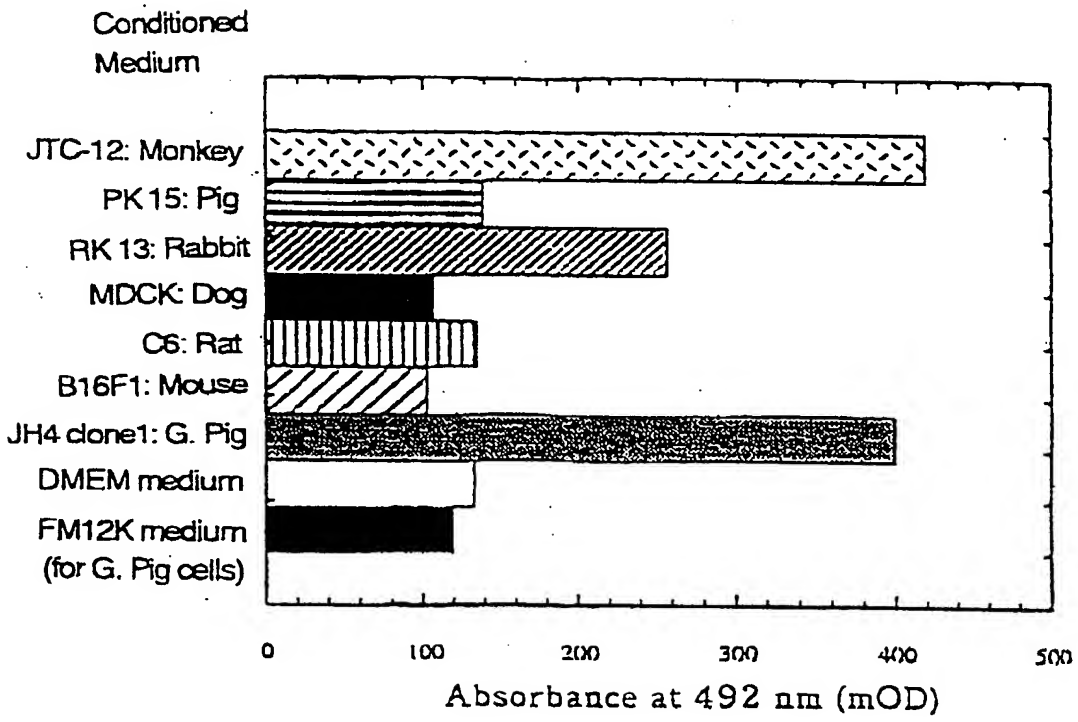


FIG. 12 B

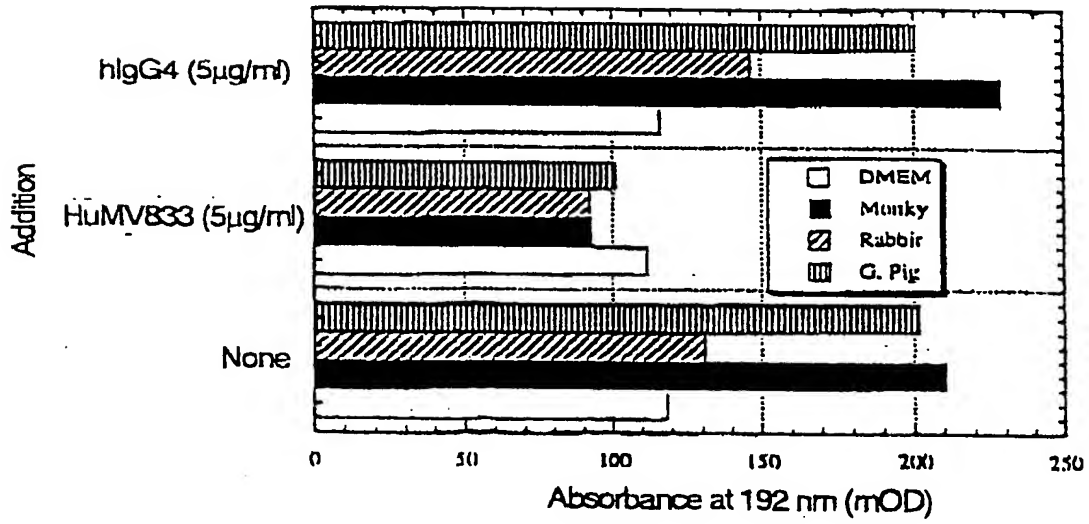
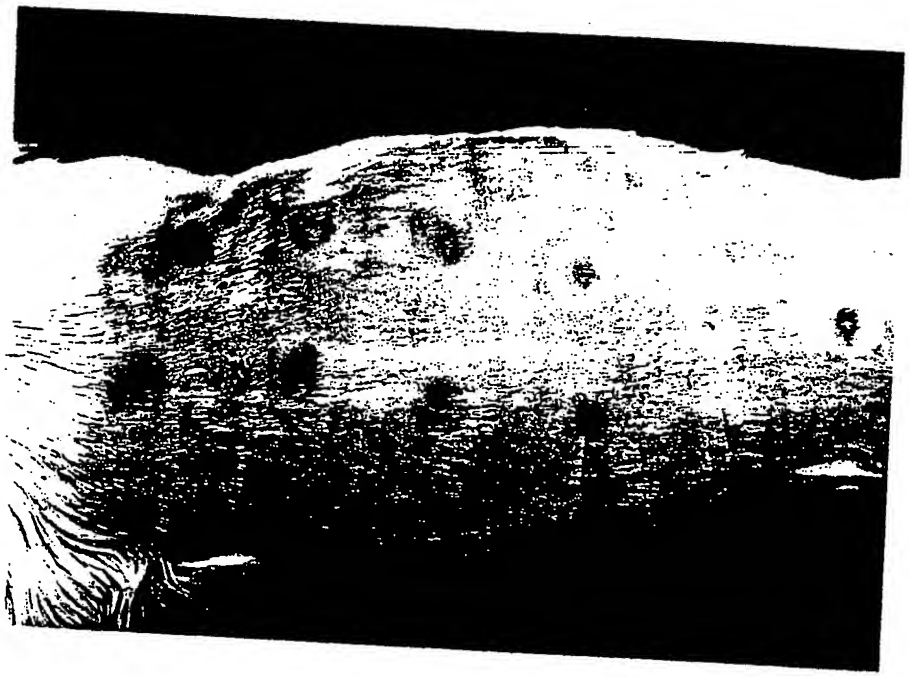


FIG. 13

None

hIgG4

HuMV833



None	①	②	③	④
hIgG4	⑤	⑥	⑦	⑧
HuMV833	⑨	⑩	⑪	⑫

FIG. 14

Synthetic oligonucleotides for light chain variable region

MV833VK1(SEQ ID NO: 9)
TATATCTAGACCACCATGACCATGTTCTCACTAGCTCTTCTCCTCAGTCTTCTGCTCCTC
TG

MV833VK2(SEQ ID NO: 10)
CGGAGACTGGGTCATCTGGATATCTGCCCTAGAATCGGAGACACAGAGGAGCAGAAGA
CTGAG

MV833VK3(SEQ ID NO: 11)
CCAGATGACCCAGTCTCCGAGCTCCCTGTCCGCATCTGTGGGAGACAGAGTCACCATC
ACTTGCATAACC

MV833VK4(SEQ ID NO: 12)
GCTTTCCCTGGCTTCTGCTGATACCAGTTCATATCATCATCAATATCATTGCTGGTTATG
CAAGTGATGGTGAC

MV833VK5(SEQ ID NO: 13)
CAGCAGAAGCCAGGGAAAGCTCCTAAGCTTCTTATTTCAGAAGGCAATACTCTTCGTCC
TGGAGTCCCATCCC

MV833VK6(SEQ ID NO: 14)
GCTGCAGGCTGCTAATTGTGAGAGTAAAATCTGTACCATAGCCACTTCCGGAGAATCG
GGATGGGACTCCAGGACG

MV833VK7(SEQ ID NO: 15)
CACAATTAGCAGCCTGCAGCCTGAAGATGTTGCAACTTACTACTGTTTCCAAAGTGATA
ACTTGCCGTAC

MV833VK8(SEQ ID NO: 16)
TATATCTAGACTACTTACGTTTTATTTCCACCTTGGTCCCTTGGCCAAACGTGTACGGCA
AGTTATCACTTTGG

FIG. 15

Synthetic oligonucleotides for heavy chain variable region

MV833VH1(SEQ ID NO: 17)
TATATCTAGACCACCATGGAATGGAGTTGGATATTTCTCTTTCTCCTGTCAGGAACTGCA
GGTGTCCACTCTGAGGTCC

MV833VH2(SEQ ID NO: 18)
CTTTGCAGGACACCTTCACTGAAGCTCCAGGCTTCTTCACCTCAGCTCCAGACTGCACC
AGTTGGACCTCAGAGTGGACACC

MV833VH3(SEQ ID NO: 19)
CAGTGAAGGTGTCCTGCAAAGCTTCTGGAGACACATTCACTACCTATGTTATACACTGG
ATGCGTCAGGCTCCTGGGCAGG

MV833VH4(SEQ ID NO: 20)
GTACTTAGTACCATCATTGTAAGGATTAATATATCCAATCCACTCGAGACCCTGCCCAG
GAGCCTGAC

MV833VH5(SEQ ID NO: 21)
CTTACAATGATGGTACTAAGTACAATGAGAAGTTCAAAGGCAGAGTCACAATCACTTCA
GACAAATCC

MV833VH6(SEQ ID NO: 22)
CGCAGTGTCCTCAGACCTCAGGCTGCTGAGCTCCATGTAGGCTGTGCTGGTGGATTTG
TCTGAAGTGATTGTG

MV833VH7(SEQ ID NO: 23)
GAGGACACTGCGGTCTATTACTGTGCAAGGATCTACTATGATTACGACGGAGACTACTG
GGGTCAAGG

MV833VH8(SEQ ID NO: 24)
TATATCTAGAGGCCATTCTTACCTGAGGAGACTGTGACCAGGGTGCCTTGACCCCAGTA
GTCTCC

FIG. 16

VL:

ETTVTQSPASLSMAIGEKVTIRC xxxx1234x567xxx8xx901x2 DIQMTQSPSSLSASVGDRVTITC	1-23:	23	12	11
WYQQKPGEPPKLLIS 1234567xx89012x WYQQKPGKVPKLLIY	35-49:	15	12	3
GVPSRFSSSGYGTDFVFTIEDILSEDVADYYC 1234567x89x0123xx45xxxxx6789x012 GVPSRFSGSGSGTDFTLTISSLQPEDVATYYC	57-88:	32	22	10
FGGGTKLEIK 12x345x678 FGQGTKVEIK	98-107:	10	8	2
**********		80	54 (67.5%)	26

FIG. 17

VH:

EVQLQQSGPELVKPGASVKMSCKASGDTFT x123x456x7xx890x123x456789x01x QVQLVQSGAEVKKPGSSVKVSCKASGGTFS	1-30:	30	21	9
WMKQKPGQGLEWIG 1xx2x3456789x0 WVRQAPGQGLEWMG	36-49:	14	10	4
KATLTSDKSSSTAYMELSSLTSEDSAVYYCAR xx1x2x345x6789012345x678x9012345 RVTITADKSTSTAYMELSSLRSEDTAVYYCAR	67-98:	32	25	7
WGQGTTLT 12345xxx WGQGTLVI	108-115:	8	5	3
		84	61 (72.6%)	23

FIG. 18

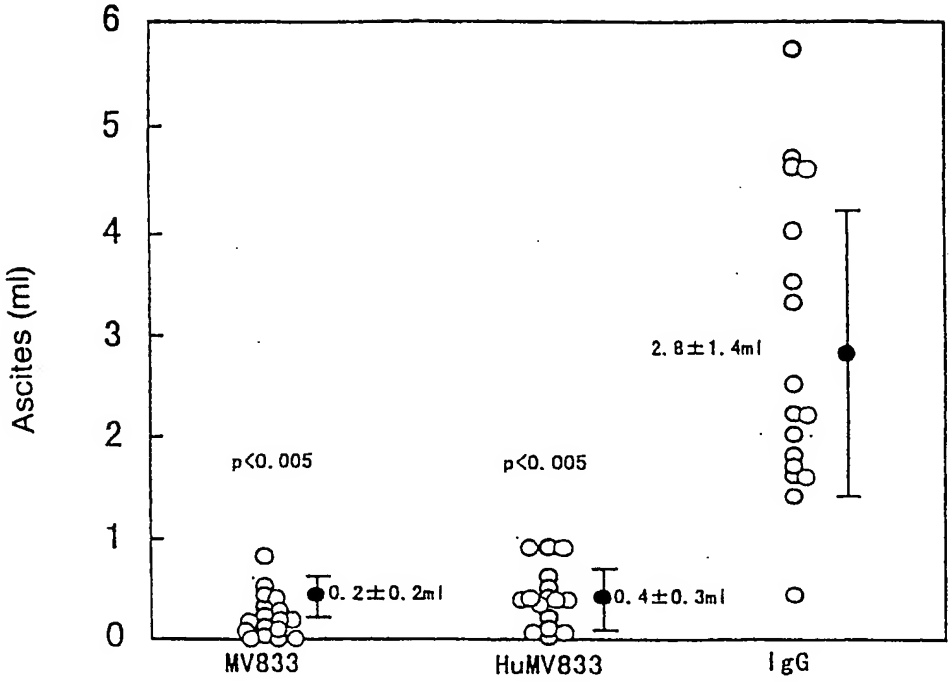


FIG. 19

IgG-administrated group (n=17) 34.1±5.3 days
HuMV833-administrated group (n=17) >47.8±7.5 days (p<0.01)
MV833-administrated group (n=17) 44.4±7.3 days (p<0.05)

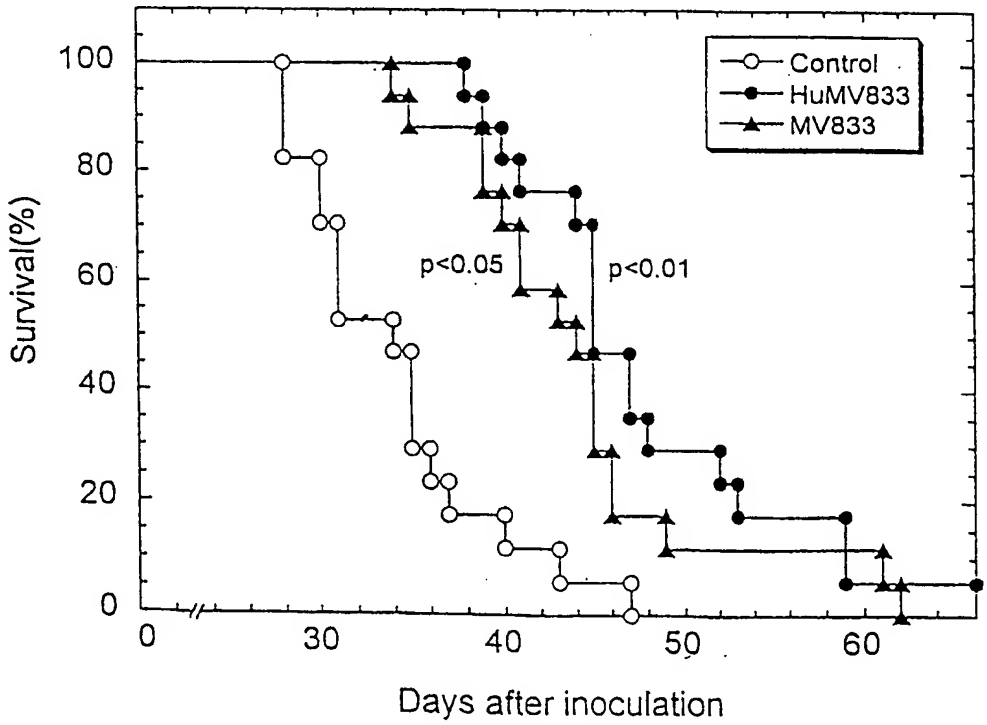


FIG. 20

HuMV833	0.63±0.71ml(n=15)	p<0.005
MV833	0.43±0.39ml(n=15)	p<0.005
IgG	2.09±1.08ml(n=15)	

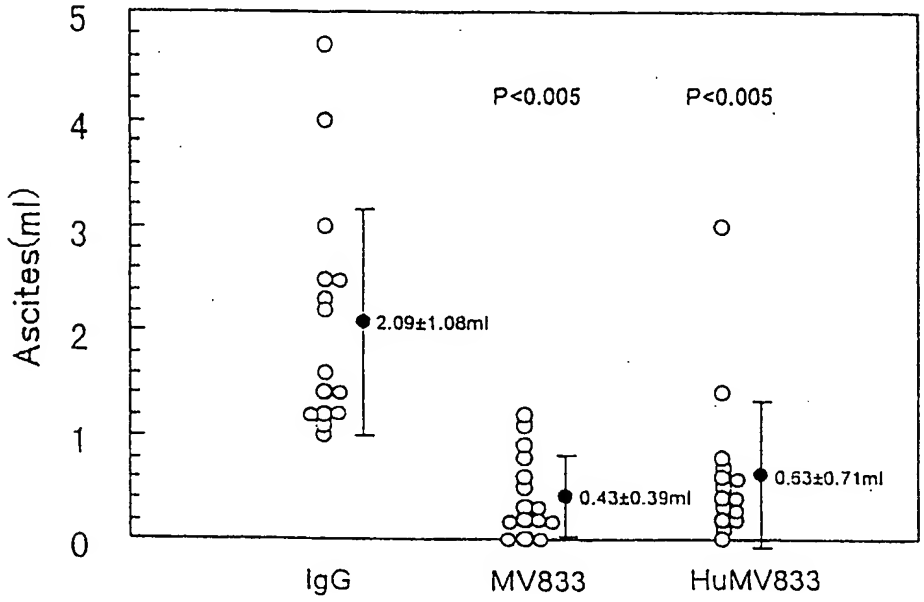


FIG. 21

IgG-administrated group (n=17)	10. 9±3. 6 days
HuMV833-administrated group (n=16)	21. 1±5. 2 days (p<0. 01)
MV833-administrated group (n=16)	20. 4±8. 2 days (p<0. 05)

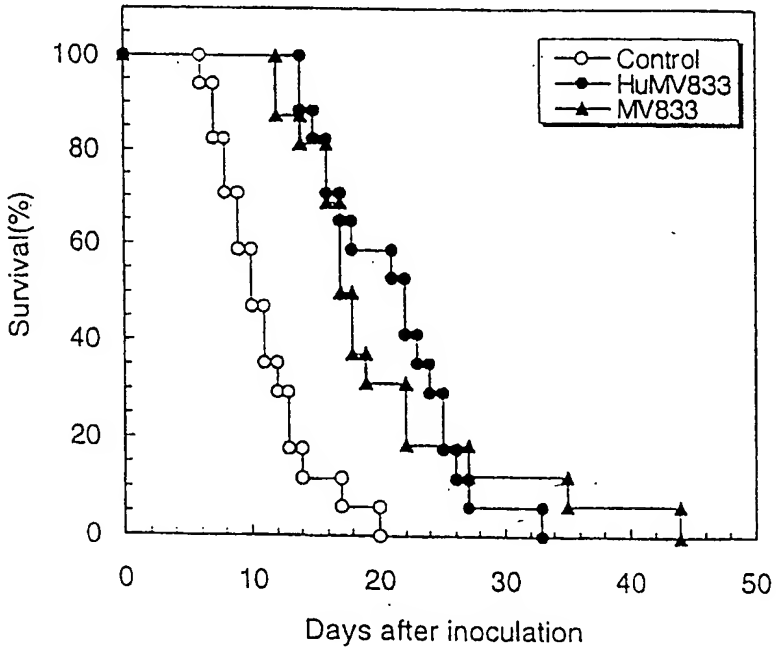


FIG. 22

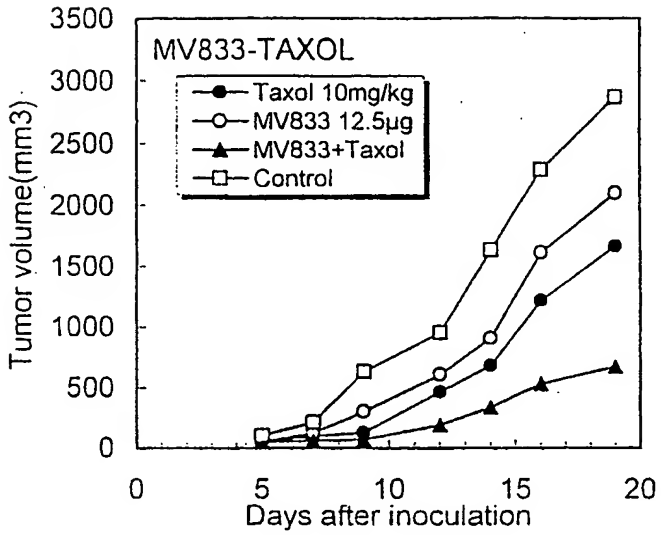
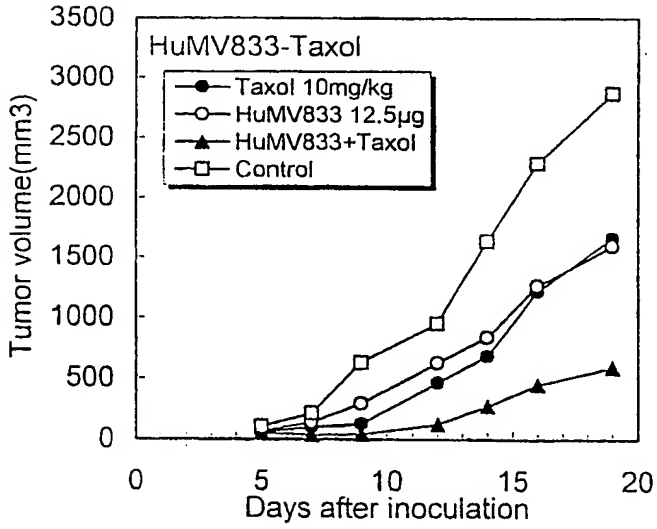

MV833-TAXOL
Taxol 10mg/kg
MV833 12.5µg
MV833+Taxol
Control
Tumor volume(mm3)
3500
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
Days after inoculation

FIG. 23


HuMV833-Taxol
Taxol 10mg/kg
HuMV833 12.5µg
HuMV833+Taxol
Control
Tumor volume(mm3)
3500
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
Days after inoculation

SEQUENCE LISTING

<110> Co, Man, Sang
Vassquez, Max
Asano, Makoto
Yukita, Ayako
Matsumoto, Tomoe
Ota, Yoshinobu
Okamoto, Masaji

<120> Humanized Monoclonal Antibodies Against Vascular Endothelial Cell Growth Factor

<130> 07898/034WO1 PH-727PCT

<140>

<141>

<160> 28

<170> PatentIn Ver. 2.0

<210> 1

<211> 387

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (1)..(387)

<220>

<221> sig_peptide

<222> (1)..(66)

<220>

<221> mat_peptide

<222> (67)..(387)

<400> 1

```
atg acc atg ttc tca cta gct ctt ctc ctc agt ctt ctg ctc ctc tgt   48
Met Thr Met Phe Ser Leu Ala Leu Leu Leu Ser Leu Leu Leu Leu Cys
        -20                 -15                 -10

gtc tcc gat tct agg gca gaa aca act gtg acc cag tct cca gca tcc   96
Val Ser Asp Ser Arg Ala Glu Thr Thr Val Thr Gln Ser Pro Ala Ser
     -5              -1   1               5                  10

ctg tcc atg gct ata gga gaa aaa gtc acc atc aga tgc ata acc agc  144
Leu Ser Met Ala Ile Gly Glu Lys Val Thr Ile Arg Cys Ile Thr Ser
                15                  20                  25

aat gat att gat gat gat atg aac tgg tac cag cag aag cca ggg gaa  192
Asn Asp Ile Asp Asp Asp Met Asn Trp Tyr Gln Gln Lys Pro Gly Glu
             30                  35                  40

cct cct aag ctc ctt att tca gaa ggc aat act ctt cgt cct gga gtc  240
Pro Pro Lys Leu Leu Ile Ser Glu Gly Asn Thr Leu Arg Pro Gly Val
         45                  50                  55
```

```
cca tcc cga ttc tcc agc agt ggc tat ggt aca gat ttt gtt ttt aca  288
Pro Ser Arg Phe Ser Ser Ser Gly Tyr Gly Thr Asp Phe Val Phe Thr
     60                  65                  70

att gaa gac ata ctc tca gaa gat gtt gca gat tac tac tgt ttc caa  336
Ile Glu Asp Ile Leu Ser Glu Asp Val Ala Asp Tyr Tyr Cys Phe Gln
 75                  80                  85                  90

agt gat aac ttg ccg tac acg ttc ggc ggg ggg acc aag ctg gaa ata  384
Ser Asp Asn Leu Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile
                 95                 100                 105

aaa                                                              387
Lys
```

<210> 2
<211> 129
<212> PRT
<213> Homo sapiens

<400> 2

```
Met Thr Met Phe Ser Leu Ala Leu Leu Leu Ser Leu Leu Leu Leu Cys
        -20                 -15                 -10
Val Ser Asp Ser Arg Ala Glu Thr Thr Val Thr Gln Ser Pro Ala Ser
     -5              -1   1                   5                  10
Leu Ser Met Ala Ile Gly Glu Lys Val Thr Ile Arg Cys Ile Thr Ser
                 15                  20                  25
Asn Asp Ile Asp Asp Asp Met Asn Trp Tyr Gln Gln Lys Pro Gly Glu
             30                  35                  40
Pro Pro Lys Leu Leu Ile Ser Glu Gly Asn Thr Leu Arg Pro Gly Val
         45                  50                  55
Pro Ser Arg Phe Ser Ser Ser Gly Tyr Gly Thr Asp Phe Val Phe Thr
     60                  65                  70
Ile Glu Asp Ile Leu Ser Glu Asp Val Ala Asp Tyr Tyr Cys Phe Gln
 75                  80                  85                  90
Ser Asp Asn Leu Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile
                 95                 100                 105
Lys
```

<210> 3
<211> 411
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(411)

<220>
<221> sig_peptide
<222> (1)..(57)

<220>

<221> mat_peptide
<222> (58)..(411)

<400> 3
atg gaa tgg agt tgg ata ttt ctc ttt ctc ctg tca gga act gca ggt 48
Met Glu Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
-15 -10 -5

gtc cac tct gag gtc caa ctg cag cag tct gga cct gag ctg gta aag 96
Val His Ser Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
-1 1 5 10

cct ggg gct tca gtg aag atg tcc tgc aag gct tct gga gac aca ttc 144
Pro Gly Ala Ser Val Lys Met Ser Cys Lys Ala Ser Gly Asp Thr Phe
15 20 25

act acc tat gtt ata cac tgg atg aag cag aag cct ggg cag ggc ctt 192
Thr Thr Tyr Val Ile His Trp Met Lys Gln Lys Pro Gly Gln Gly Leu
30 35 40 45

gag tgg att gga tat att aat cct tac aat gat ggt act aag tac aat 240
Glu Trp Ile Gly Tyr Ile Asn Pro Tyr Asn Asp Gly Thr Lys Tyr Asn
50 55 60

gag aag ttc aaa ggc aag gcc aca ctg act tca gac aaa tcc tcc agc 288
Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Ser Asp Lys Ser Ser Ser
65 70 75

aca gcc tac atg gag ctc agc agc ctg acc tct gag gac tct gcg gtc 336
Thr Ala Tyr Met Glu Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
80 85 90

tat tac tgt gca agg atc tac tat gat tac gac ggg gac tac tgg ggc 384
Tyr Tyr Cys Ala Arg Ile Tyr Tyr Asp Tyr Asp Gly Asp Tyr Trp Gly
95 100 105

caa ggc acc act ctc aca gtc tcc tca 411
Gln Gly Thr Thr Leu Thr Val Ser Ser
110 115

<210> 4
<211> 137
<212> PRT
<213> Homo sapiens

<400> 4
Met Glu Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
-15 -10 -5
Val His Ser Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
-1 1 5 10
Pro Gly Ala Ser Val Lys Met Ser Cys Lys Ala Ser Gly Asp Thr Phe
15 20 25

```
Thr Thr Tyr Val Ile His Trp Met Lys Gln Lys Pro Gly Gln Gly Leu
 30                  35                  40                  45
Glu Trp Ile Gly Tyr Ile Asn Pro Tyr Asn Asp Gly Thr Lys Tyr Asn
                 50                  55                  60
Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Ser Asp Lys Ser Ser Ser
             65                  70                  75
Thr Ala Tyr Met Glu Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
         80                  85                  90
Tyr Tyr Cys Ala Arg Ile Tyr Tyr Asp Tyr Asp Gly Asp Tyr Trp Gly
     95                  100                 105
Gln Gly Thr Thr Leu Thr Val Ser Ser
110                 115
```

<210> 5
<211> 387
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(387)

<220>
<221> sig_peptide
<222> (1)..(66)

<220>
<221> mat_peptide
<222> (67)..(387)

<400> 5

```
atg acc atg ttc tca cta gct ctt ctc ctc agt ctt ctg ctc ctc tgt   48
Met Thr Met Phe Ser Leu Ala Leu Leu Leu Ser Leu Leu Leu Leu Cys
        -20                 -15                 -10

gtc tcc gat tct agg gca gat atc cag atg acc cag tct ccg agc tcc   96
Val Ser Asp Ser Arg Ala Asp Ile Gln Met Thr Gln Ser Pro Ser Ser
     -5              -1   1               5                  10

ctg tcc gca tct gtg gga gac aga gtc acc atc act tgc ata acc agc  144
Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Ile Thr Ser
                 15                  20                  25

aat gat att gat gat gat atg aac tgg tat cag cag aag cca ggg aaa  192
Asn Asp Ile Asp Asp Asp Met Asn Trp Tyr Gln Gln Lys Pro Gly Lys
             30                  35                  40

gct cct aag ctt ctt att tca gaa ggc aat act ctt cgt cct gga gtc  240
Ala Pro Lys Leu Leu Ile Ser Glu Gly Asn Thr Leu Arg Pro Gly Val
         45                  50                  55

cca tcc cga ttc tcc gga agt ggc tat ggt aca gat ttt act ctc aca  288
Pro Ser Arg Phe Ser Gly Ser Gly Tyr Gly Thr Asp Phe Thr Leu Thr
     60                  65                  70
```

```
att agc agc ctg cag cct gaa gat gtt gca act tac tac tgt ttc caa  336
Ile Ser Ser Leu Gln Pro Glu Asp Val Ala Thr Tyr Tyr Cys Phe Gln
 75                  80                  85                  90

agt gat aac ttg ccg tac acg ttt ggc caa ggg acc aag gtg gaa ata  384
Ser Asp Asn Leu Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu Ile
                 95                 100                 105

aaa                                                              387
Lys
```

<210> 6
<211> 129
<212> PRT
<213> Homo sapiens

<400> 6

```
Met Thr Met Phe Ser Leu Ala Leu Leu Leu Ser Leu Leu Leu Leu Cys
        -20                 -15                 -10
Val Ser Asp Ser Arg Ala Asp Ile Gln Met Thr Gln Ser Pro Ser Ser
     -5              -1   1                5                  10
Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Ile Thr Ser
                 15                  20                  25
Asn Asp Ile Asp Asp Asp Met Asn Trp Tyr Gln Gln Lys Pro Gly Lys
             30                  35                  40
Ala Pro Lys Leu Leu Ile Ser Glu Gly Asn Thr Leu Arg Pro Gly Val
         45                  50                  55
Pro Ser Arg Phe Ser Gly Ser Gly Tyr Gly Thr Asp Phe Thr Leu Thr
     60                  65                  70
Ile Ser Ser Leu Gln Pro Glu Asp Val Ala Thr Tyr Tyr Cys Phe Gln
 75                  80                  85                  90
Ser Asp Asn Leu Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu Ile
                 95                 100                 105
Lys
```

<210> 7
<211> 411
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(411)

<220>
<221> sig_peptide
<222> (1)..(57)

<220>
<221> mat_peptide
<222> (58)..(411)

<400> 7

```
atg gaa tgg agt tgg ata ttt ctc ttt ctc ctg tca gga act gca ggt   48
Met Glu Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
                -15                 -10                 -5

gtc cac tct gag gtc caa ctg gtg cag tct gga gct gag gtg aag aag   96
Val His Ser Glu Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
         -1   1               5                  10

cct gga gct tca gtg aag gtg tcc tgc aaa gct tct gga gac aca ttc  144
Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Asp Thr Phe
     15                  20                  25

act acc tat gtt ata cac tgg atg cgt cag gct cct ggg cag ggt ctc  192
Thr Thr Tyr Val Ile His Trp Met Arg Gln Ala Pro Gly Gln Gly Leu
 30                  35                  40                  45

gag tgg att gga tat att aat cct tac aat gat ggt act aag tac aat  240
Glu Trp Ile Gly Tyr Ile Asn Pro Tyr Asn Asp Gly Thr Lys Tyr Asn
                50                  55                  60

gag aag ttc aaa ggc aga gtc aca atc act tca gac aaa tcc acc agc  288
Glu Lys Phe Lys Gly Arg Val Thr Ile Thr Ser Asp Lys Ser Thr Ser
            65                  70                  75

aca gcc tac atg gag ctc agc agc ctg agg tct gag gac act gcg gtc  336
Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val
         80                  85                  90

tat tac tgt gca agg atc tac tat gat tac gac gga gac tac tgg ggt  384
Tyr Tyr Cys Ala Arg Ile Tyr Tyr Asp Tyr Asp Gly Asp Tyr Trp Gly
     95                  100                 105

caa ggc acc ctg gtc aca gtc tcc tca                              411
Gln Gly Thr Leu Val Thr Val Ser Ser
110                 115
```

<210> 8
<211> 137
<212> PRT
<213> Homo sapiens

<400> 8

```
Met Glu Trp Ser Trp Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
                -15                 -10                 -5
Val His Ser Glu Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
         -1   1               5                  10
Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Asp Thr Phe
     15                  20                  25
Thr Thr Tyr Val Ile His Trp Met Arg Gln Ala Pro Gly Gln Gly Leu
 30                  35                  40                  45
Glu Trp Ile Gly Tyr Ile Asn Pro Tyr Asn Asp Gly Thr Lys Tyr Asn
                50                  55                  60
Glu Lys Phe Lys Gly Arg Val Thr Ile Thr Ser Asp Lys Ser Thr Ser
            65                  70                  75
```

Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val
80 85 90
Tyr Tyr Cys Ala Arg Ile Tyr Tyr Asp Tyr Asp Gly Asp Tyr Trp Gly
95 100 105
Gln Gly Thr Leu Val Thr Val Ser Ser
110 115

<210> 9
<211> 79
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides
<400> 9
tatatctaga ccaccatgga atggagttgg atatttctct ttctcctgtc aggaactgca 60
ggtgtccact ctgaggtcc 79

<210> 10
<211> 82
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 10

ctttgcagga caccttcact gaagctccag gcttcttcac ctcagctcca gactgcacca 60
gttggacctc agagtggaca cc 82

<210> 11
<211> 81
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 11
cagtgaaggt gtcctgcaaa gcttctggag acacattcac tacctatgtt atacactgga 60
tgcgtcaggc tcctgggcag g 81

<210> 12
<211> 68
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 12
gtacttagta ccatcattgt aaggattaat atatccaatc cactcgagac cctgcccagg 60
agcctgac 68

<210> 13
<211> 68
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 13
cttacaatga tggtactaag tacaatgaga agttcaaagg cagagtcaca atcacttcag 60
acaaatcc 68

<210> 14
<211> 73
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 14
cgcagtgtcc tcagacctca ggctgctgag ctccatgtag gctgtgctgg tggatttgtc 60
tgaagtgatt gtg 73

<210> 15
<211> 68
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 15

gaggacactg cggtctatta ctgtgcaagg atctactatg attacgacgg agactactgg 60
ggtcaagg 68

<210> 16
<211> 65
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 16
tatatctaga ggccattctt acctgaggag actgtgacca gggtgccttg accccagtag 60
tctcc 65

<210> 17

<211> 62
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 17
tatatctaga ccaccatgac catgttctca ctagctcttc tcctcagtct tctgctcctc 60
tg 62

<210> 18
<211> 63
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 18
cggagactgg gtcatctgga tatctgccct agaatcggag acacagagga gcagaagact 60
gag 63

<210> 19
<211> 70
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 19
ccagatgacc cagtctccga gctccctgtc cgcatctgtg ggagacagag tcaccatcac 60
ttgcataacc 70

<210> 20
<211> 73
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 20
cagcagaagc cagggaaagc tcctaagctt cttatttcag aaggcaatac tcttcgtcct 60
ggagtcccat ccc 73

<210> 21
<211> 73
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 21
cagcagaagc cagggaaagc tcctaagctt cttatttcag aaggcaatac tcttcgtcct 60
ggagtcccat ccc 73

<210> 22
<211> 76
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 22
gctgcaggct gctaattgtg agagtaaaat ctgtaccata gccacttccg gagaatcggg 60
atgggactcc aggacg 76

<210> 23
<211> 70
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 23
cacaattagc agcctgcagc ctgaagatgt tgcaacttac tactgtttcc aaagtgataa 60
cttgccgtac 70

<210> 24
<211> 74
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 24
tatatctaga ctacttacgt tttatttcca ccttggtccc ttggccaaac gtgtacggca 60
agttatcact ttgg 74

<210> 25
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 25
tatatctaga attcccccccc cccccccccc 30

<210> 26
<211> 46

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 26
tatagagctc aagcttggat ggtgggaaga tggatacagt tggtgc 46

<210> 27
<211> 50
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic nucleotides

<400> 27
tatagagctc aagcttccag tggatagacc gatggggctg tcgttttggc 50

<210> 28
<211> 121
<212> PRT
<213> Homo sapiens

<400> 28

Ala	Pro	Met	Ala	Glu	Gly	Gly	Gly	Gln	Asn	His	His	Glu	Val	Val	Lys
1				5				10					15		
Phe	Met	Asp	Val	Tyr	Gln	Arg	Ser	Tyr	Cys	His	Pro	Ile	Glu	Thr	Leu
		20					25					30			
Val	Asp	Ile	Phe	Gln	Glu	Tyr	Pro	Asp	Glu	Ile	Glu	Tyr	Ile	Phe	Lys
	35					40					45				
Pro	Ser	Cys	Val	Pro	Leu	Met	Arg	Cys	Gly	Gly	Cys	Cys	Asn	Asp	Glu
50					55					60					
Gly	Leu	Glu	Cys	Val	Pro	Thr	Glu	Glu	Ser	Asn	Ile	Thr	Met	Gln	Ile
65				70					75					80	
Met	Arg	Ile	Lys	Pro	His	Gln	Gly	Gln	His	Ile	Gly	Glu	Met	Ser	Phe
			85					90					95		
Leu	Gln	His	Asn	Lys	Cys	Glu	Cys	Arg	Pro	Lys	Lys	Asp	Arg	Ala	Arg
		100					105					110			
Gln	Glu	Asn	Pro	Cys	Gly	Pro	Cys	Ser							
	115					120									

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/29415

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/29415

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/387.3, 387.7, 388.3, 388.85; 536/23.53; 435/69.6, 4, 7.1, 326, 328, 536, 252.1; 424/133.1, 156.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS, MEDLINE, USPATFUL
search terms: humanized, VEGF, vascular endothelial cell growth factor, MV833, chimeric, CDR grafted, angiogenesis, cancer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PRESTA et al. Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Research. 15 October 1997, Vol. 57, pages 4593-4599, see abstract.	1
X --- Y	WO 98/45331 A2 (GENENTECH, INC.) 15 October 1998 (15/10/1998), see page 2, 11, 15, 19, 36, 39, 41, 43, and 44-47.	1 ------- 2, 10-17, 21-33, 41-47, 49-53, and 55-60

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 22 MARCH 2000	Date of mailing of the international search report 25 APR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer LARRY HELMS Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/29415

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

C07K 16/30; C12N 15/11, 15/85, 1/20; G01N 33/50; A61K 39/395, 45/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/387.3, 387.7, 388.3, 388.85; 536/23.53; 435/69.6, 4, 7.1, 326, 328, 336, 252.1; 424/133.1, 156.1

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-16 and 26, drawn to an antibody.
Group II, claims 17-25, drawn to a polynucleotide, vector, and host cell.
Group III, claims 27-42, drawn to a method for inhibiting an angiogenesis-associated disorder.
Group IV, claims 43-48, drawn to a method of increasing the mean progression-free survival time of a subject.
Group V, claims 49-54, drawn to a method of increasing the objective response rate of a subject.
Group VI, claims 55-58, drawn to a method of diagnosing an angiogenesis-associated disorder.
Group VII, claims 59-60, drawn to a method for determining the prognosis of a subject.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. If any of groups I or III is elected, then an election of species between the following species is required. The species are as follows:

Species A: LC49
Species B: LC67
Species C: HC1
Species D: HC27
Species E: HC30
Species F: HC37
Species G: HC48
Species H: HC72
Species I: LC1
Species J: LC2
Species K: LC3
Species L: LC4
Species M: LC43
Species N: LC67
Species O: HC16
Species P: HC116
Species Q: HC115

The claims are deemed to correspond to the species listed above in the following manner:

All claims in Groups I and III are generic.

The following claims are generic: 1-16, 26, and 27-42.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. If either of Groups III or VI are elected, then an election of species is required. The species are as follows:

Species R: Cancer
Species S: solid tumor

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/29415

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KANAI et al. Anti-tumor and anti-metastatic effects of human-vascular-endothelial-growth-factor-neutralizing antibody on human colon and gastric carcinoma xenotransplanted orthotopically into nude mice. Int. J. Cancer. 11 October 1998, Vol. 77, pages 933-936, especially pages 933 and 935.	1-2, 10-17, 21-33, 41-47, 49-53, and 55-60

Species T: diabetic retinopathy
Species U: muscular degeneration
Species V: Kaposi sarcoma
Species W: chronic articular rheumatism
Species X: psoriasis
Species Y: angioma
Species Z: scleroderma
Species AA: neovascular glaucoma

The claims are deemed to correspond to the species listed above in the following manner:

claims 27-42

The following claims are generic: 27-29, 31-42, 55-57

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
Group I recites the technical feature of a humanized VEGF antibody (See claim 1). As evidenced by Rockwell et al (US Patent 5,840,301, issued 24 November 1998), humanized VEGF antibodies are not novel and do lack an inventive step, thus, the technical feature of Group I is not special. Therefore, the claims are not so linked as to form a single inventive concept under PCT Rule 13.1. Accordingly the following lack of unity is put forth.

Group I recites the technical feature of a humanized antibody to VEGF, Group II recites the technical feature of a polynucleotide. Group III recites the technical feature of a method of inhibiting an angiogenesis-associated disorder. Group IV recites the technical feature of increasing the median progression-free survival time of a subject. Group V recites the technical feature of a method of increasing the objective response rate of a subject. Group VI recites the technical feature of a method of diagnosing an angiogenesis-associated disorder. Group VII recites the technical feature of a method for determining the prognosis of a subject. Thus, inventions of Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1.

The species listed as A-Q above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: Each species in claims 4-6, 8-9, 35-37, and 39-40 is distinct for an amino acid position in the light or heavy chain and each have a structural feature that makes each distinct. Each would impart unique features and influence binding of antigen, affect half life, affect pharmokinetics, would have an affect on folding of the polypeptide, and affect the functioning of the antibody. In addition, art on one species would not be art on the other species. Thus, the species listed as A-Q above do not relate to a single inventive concept under PCT Rule 13.1.

The species listed as R-AA above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: Each species listed as R-AA in claims 29 and 57 is distinct in having affects on different organs in the body, different methods of diagnosis, and different prognosis. In addition, art on one would not be art on the others. Thus, the species listed as R-AA above do not relate to a single inventive concept under PCT Rule 13.1.